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Sediments as a dispersal vector of aquatic invertebrates: An estimation of propagule pressure associated with 'no ballast on board' vessels

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Sediments as a dispersal vector of aquatic invertebrates: an estimation of propagule pressure associated with 'no ballast on board' vessels

by

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ABSTRACT

Ballast water has been the primary vector of nonindigenous species (NIS) to the Laurentian Great Lakes over the past 45 years. Although ballast water exchange regulations were implemented in 1993 to reduce propagule loads, new NIS continue to be discovered. A possible explanation for this trend is the importance of alternative vectors, such as resting stages of invertebrates in residual ballast sediments of transoceanic ships claiming 'no ballast on board'. To determine the risk of invasion potentially associated with this vector, I collected sediment samples from 39 ships entering the Great Lakes and measured the density, diversity and viability of resting stages contained therein. Viable resting stages of NIS were found in 32% of ships, at a median density of 3.0×10^5 ship^{-1}. Twenty-one NIS were identified, consisting exclusively of rotifers and cladocerans. I subsequently conducted *in situ* experiments using emergence traps to assess the introduction potential of invertebrate diapausing stages present in ships' ballast sediment. Hatching was observed on all four ships, although not from all sediments. Overall hatch rates were very low (0.5 individuals per 500 g sediment), typically involving activation of <0.05% of total eggs present. While dormancy is a characteristic enabling enhanced survival during transportation, it becomes an impediment for introduction as resting stages that are buried in sediments appear to have little chance for expulsion from ballast tanks. Results from this study indicate, however, that diapausing eggs contained in ballast sediment of NOBOB ships are a potential mechanism for introduction of new NIS to the Great Lakes.

This dissertation is dedicated to my husband, Dave;

without your patience, encouragement and support, I could not have endured the rigours and stress of graduate school

and

to Jessica, Tyler and Kaitlyn,

who remind me how many questions are yet unanswered.

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STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution. I would like to thank the American Society of Limnology and Oceanography and Blackwell Publishing Ltd. for their permission to include as Chapter II and V the articles:

Bailey, S.A., Duggan, I.C., van Overdijk, C.D.A., Jenkins, P.T. and MacIsaac, H.J. (2003) Viability of invertebrate diapausing eggs collected from residual ballast sediment. Limnology and Oceanography 48: 1701-1710,

and

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respectively. Two other chapters, III and IV, were reprinted from journal articles, but I held copyrights at the time of the dissertation. They are:

Bailey, S.A., Duggan, I.C., Jenkins, P.T. and MacIsaac, H.J. (2005) Invertebrate resting stages in residual ballast sediment of transoceanic ships. Canadian Journal of Fisheries and Aquatic Sciences (in press)

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Chapters published, or accepted for publication, in the above journals have been modified slightly for consistency.

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GENERAL INTRODUCTION

Freshwater biodiversity, broadly defined as the variety and variability among living organisms and the ecological complexes in which they occur, is affected by both biotic and abiotic factors (Brönmark and Hansson 2002). Changes in land use, biotic exchange and climate change are recognized as the major drivers of biodiversity change for freshwater ecosystems globally; among lakes, introduction of nonindigenous species (NIS) is expected to be the leading cause of biodiversity change for the next 25 to 100 years (Sala et al. 2000; Brönmark and Hansson 2002).

Although the introduction of NIS to new ecosystems often appears to occur with little impact on the recipient community, approximately 5-20% of established invaders are predicted to have profound effects on the physical, chemical and/or biological parameters of the host environment (Williamson and Fitter 1996; Ricciardi and Atkinson 2004). Invasions may even facilitate further invasions (Simberloff and von Holle 1999; Ricciardi 2001). For example, the invasion by the zebra mussel, *Dreissena polymorpha*, has had profound impact on the water quality and ecology of the Laurentian Great Lakes (MacIsaac 1999). By filtering suspended sediment, microzooplankton, phytoplankton and bacteria from the water column, zebra mussels altered the physical environment by enhancing water clarity such that submerged aquatic plant growth increased in shallow areas; these changes in turn altered native fish and invertebrate communities, and possibly facilitated the establishment of nonindigenous amphipods and gobies (see MacIsaac 1996; Ricciardi 2001).

In addition to ecological impacts, NIS may impart devastating economic and/or health impacts. Pimentel et al. (2000) estimated direct damage and control costs of NIS in the United States at US$136.63 billion annually, while characterised costs associated with ten NIS in Canada totalled CDN$187 million per year (Colautti et al. 2005b). Economic costs often result from direct damage and control costs associated with the NIS, or as an indirect by-product of its presence (Colautti et al. 2005b). For example, zebra mussel fouling of intake pipes resulted in direct costs for power generation plants on the Great Lakes that had to implement expensive antifouling systems; municipal water plants suffered indirect costs when forced to install charcoal filters to reduce the unpleasant taste generated by geosmin, a compound generated by macrophytes which grew in profusion as a result of zebra mussel-induced increases in water clarity (Colautti et al. 2005b; see also MacIsaac et al. 2002a). Although it is currently impossible to accurately quantify total economic costs associated with zebra mussels in the U.S. or Canada, it has been reported that Ontario Power Generation has spent CDN$12 million for installation of chlorination equipment, with additional operating and maintenance costs of CDN$6.4 million per year (Grodowitz 2000; Wiancko 2000). In addition, Colautti et al. (2005b) estimated that Great Lakes' industries, municipalities and residents have spent as much as CDN$53.4 million for filtration and treatment systems, in addition to annual maintenance fees.

Biological invasions can also impart major economic costs on recipient communities through damage to, or consumption of, economically important indigenous species. Invaders like the European green crab (*Carcinus maenas*),

leafy spurge (*Euphorbia esula*), and white pine blister rust (*Cronartium ribicola*) decrease annual profits to aquaculture, agriculture and forestry industries, respectively, across North America. In Canada alone, these industries are valued at CDN$66 billion per year — even a modest impact of a 20% decline in profits would result in losses of CDN$13 billion annually to the Canadian economy (Colautti et al. 2005b). Similar impacts of NIS are experienced by industries around the world. For example, it has been estimated that the invasion of the comb-jelly *Mnemiopsis leidyi* in the Black Sea resulted in annual losses of nearly US$17 million to the commercial anchovy fisheries in Bulgaria, Georgia, Romania, the Russian Federation, Turkey and the Ukraine (Knowler and Barbier 2000).

Biological invasions are also evident in transmission of foreign pathogenic viruses to humans, animals and plants. Recent outbreaks of severe acute respiratory syndrome (SARS) and West Nile virus in Canada provide vivid examples of nonindigenous diseases that threaten human welfare and our economical system. Increases in global movement of humans and international trade have been major causative factors in the spread of nonindigenous infectious diseases. For example, the worldwide pattern of bubonic plague outbreaks in port areas during the late nineteenth century suggests that the disease was probably introduced by fleas on Norway rats (*Rattus norvegicus*) that were transported by vessels, whereas recent transport of NIS of mosquitoes in tire shipments have transmitted human diseases including dengue fever (Lounibos 2002). The global movement of ballast water provides perhaps the

best opportunity for transfer of entire biological communities from one region to another, including human pathogens *Vibrio cholerae*, *Cryptosporidium* sp. and *Giardia* sp. (e.g., Knight et al. 1999; Drake et al. 2001).

Stages of an Invasion

The invasion process can be divided into four basic stages: transport, introduction, establishment and spread. At each stage physical, chemical and/or biological barriers serve to 'filter' the number of species surviving to the next stage (Fig. 1.1). Early invasion studies focused principally on the establishment and spread stages of invasions, as well as properties of recipient communities and characteristics of the invaders in particular (see Crawley 1987; Lodge 1993b). For example, in a pioneering compendium involving hundreds of case studies, Elton (1958) described the invasion process in terms of niche theory. Specifically, Elton (1958) identified communities with low native diversity, those 'disturbed' by humans, and island ecosystems, as being most susceptible to and impacted by invaders. According to Elton, each of these systems was particularly vulnerable to invasion owing to the absence or reduced effectiveness of 'biotic resistance', thereby opening niche space for invading species. These concepts remain controversial to this day, as much of the literature linking diversity or stability with invasibility has not properly accounted for confounding factors (Lodge 1993b; Levine and D'Antonio 1999; Colautti and MacIsaac 2004; Levine et al. 2004; but see Zavaleta and Hulvey 2004).

Numerous recent studies have determined that invasion success is affected more by dispersal opportunity than by the composition of the recipient community or species-specific attributes (e.g., Moyle and Light 1996; Duncan et al. 2003; Rouget and Richardson 2003). One could argue that if a species cannot access a new habitat, then the physical, chemical and/or biological components of the recipient region are irrelevant. The 'propagule pressure' hypothesis, which relates invasion success to the number of release events and number of propagules per release event was founded in early studies examining the effect of release size on the probability of establishment of biological control agents (e.g., Hopper and Roush 1993; Grevstad 1999a,b; Shea and Possingham 2000). The hypothesis is based on the reasoning that as propagule pressure increases, the probability of extinction via demographic and environmental stochasticity declines (Sax and Brown 2000; Forsyth and Duncan 2001). A positive relationship between propagule pressure and invasion success has been supported in studies of a wide array of taxa, including insects, ungulates, birds, fishes, and trees (Veltman et al. 1996; Williamson 1996; Forsyth and Duncan 2001; Duncan et al. 2003; Rouget and Richardson 2003; Duggan et al. 2005).

More than one thousand NIS have been reported from marine, estuarine and freshwater habitats in North America, Europe and Australia (Ruiz et al. 1997; Olenin et al. 2000; Rigby and Taylor 2001; Grigorovich et al. 2003b,c). The mechanisms behind introduction of NIS to aquatic ecosystems are numerous, and include (but are not limited to) deliberate stocking of vertebrate and invertebrate taxa (e.g., Mills et al. 1993; Cohen and Carlton 1998), unintentional

releases of non-target species in aquaculture and deliberate releases of aquarium species (e.g., Duggan et al. 2005; Rixon et al. 2005), and construction of canals to link previously isolated water bodies (e.g., Galil 2000; Grigorovich et al. 2002). However, ballast water, hull fouling, and possibly ballast sediments have been considered the principal vector to coastal port areas and to the Great Lakes since the early 1900s (e.g., Carlton 1985, 1989; Carlton and Geller 1993; Ruiz et al. 2000; Leppäkoski et al. 2002; Grigorovich et al. 2002, 2003a; Hayes and Sliwa 2003). Although ship-mediated invasions were suspected as early as 1903, the great scale of the problem was not seriously considered until Carlton (1985) outlined the process of, and evidence for, ballast water transport. The literature was soon flooded with publications documenting the taxonomic composition of ballast water, as well as outlining patterns, mechanisms and consequences of ballast-mediated invasions to freshwater and marine ports around the world (e.g., Carlton 1989, 1996; Hallegraeff and Bolch 1991, 1992; Locke et al. 1993; Ruiz et al. 1997, 2000).

The Great Lakes have an extensive history of biological invasions, dating back to the 1830s (Mills et al. 1993). During the pre-industrial period (1881-1920), the accidental escape of cultivated plants from ornamental gardens and agriculture was the dominant vector (37%) for the introduction of NIS to the Great Lakes (Mills et al. 1993). Other historically important vectors to the Great Lakes basin have been deliberate and incidental releases associated with the aquaculture and aquarium industries (Mills et al. 1993; Rixon et al. 2005; Duggan et al. 2005). Ship-related mechanisms (solid ballast, ballast water and possibly

hull fouling) have been an active transport vector in the Great Lakes since the 1800s; however, the relative importance of shipping activities increased two-fold after the opening of the St. Lawrence Seaway provided large transoceanic vessels direct access to the lakes in 1959 (Mills et al. 1993; Grigorovich et al. 2003a; Holeck et al. 2004). Additional factors potentially contributing to the increased importance of this vector include physicochemical and biotic changes in donor and recipient ports, connection to new donor regions, and increases in ship volume and speed (Carlton 1996). For example, improvements in water quality of harbours likely resulted in greater biodiversity of planktonic communities available for uptake into ballast tanks, while decreases in transoceanic transit times may allow a larger number of entrained taxa to survive the voyage.

Ship ballast acts as a vector of NIS introductions if resident species can survive the physical, chemical and biological rigours of long-distance transport. For example, the species must tolerate periods of darkness, reduced food supply, and changing temperature, oxygen and salinity regimes for one to two weeks or more (Carlton 1985). Alternatively, taxa with cysts or dormant stages (e.g., cladoceran ephippia and dinoflagellate cysts), and those with non-feeding larval stages (e.g., barnacle cyprids) should have enhanced survival during transport in ballast sediments, and thus pose a greater invasion risk than species that lack these capabilities (Carlton 1985; Pollutech Environmental Ltd. 1992; Hallegraeff 1998). In point of fact, fifteen of the nineteen crustacean invaders that

have successfully established in the Great Lakes are known, or alleged, to produce a dormant life history stage (Bailey et al. in review).

A report by Bio-Environmental Services Ltd. to Environment Canada, which was published in 1981, speculated that zebra mussels and many other NIS could invade the Great Lakes via ships' ballast water unless effective management procedures were implemented (see Wiley 1995). However, regulations pertaining to untreated ballast water were not implemented until 1989, when Canada promulgated guidelines for the control of ballast water discharges, in response to the successful invasion by the zebra mussel. These voluntary guidelines recommended that freshwater ballast water be exchanged with open ocean water, taken in waters at least 2000 m deep and 200 nautical miles from shore, prior to entering the system. Regulations were subsequently made mandatory by the United States Coast Guard in 1993 (USCG 1993). Ballast water exchange was implemented to reduce the number of viable freshwater organisms contained within ballast water by flushing taxa into the ocean and replacing them with a much lower diversity and abundance of saltwater taxa, which presumably could not tolerate the abiotic conditions of the Great Lakes. Residual freshwater species in ballast tanks are assumed to be killed by exposure to salt water, although ballast exchange efficiency has never been tested for the Great Lakes.

Despite ballast exchange regulations, the rate of report of new invertebrate and protozoan invasions appears to have increased three-fold since 1989 (Grigorovich et al. 2003a), indicating that these regulations may be inadequate and/or that alternate vectors — including ship-related vectors other than ballast

water — are operational. Increased sampling effort and time lags between establishment and discovery of NIS may also partially account for this pattern (Costello and Solow 2003; Grigorovich et al. 2003a), although modeling exercises indicated that ballast water exchange offers incomplete protection and should be least successful for species with benthic or dormant stages contained within ballast sediments (MacIsaac et al. 2002b). In addition, many of the species detected in the lakes during the 1990s are large or otherwise conspicuous, thereby arguing against the sampling effort hypothesis.

Ballast water exchange regulations apply only to ships entering the Great Lakes system with fully-loaded ballast tanks. A second category of vessels, officially classified as 'no ballast on board' (NOBOB), are exempt from ballast water regulations since their ballast tanks are considered 'empty' (USCG 1993). However, most vessels cannot completely empty their ballast tanks due to structural and operational limitations, and carry an average of 50 tonnes of unpumpable water and 10 tonnes of ballast sediments while operating on the Great Lakes (P.T. Jenkins, Marine Consulting and Management Services, unpubl.). MacIsaac et al. (2002b) estimated that residual water in individual NOBOB vessels could carry up to 10^6 zooplankton and orders of magnitude more bacteria, depending on the proximity of the ballast water source to the Great Lakes. As NOBOB vessels dominate trade into the Great Lakes (Colautti et al. 2003), the cumulative invasion risk posed by NOBOB vessels appears far greater than ships conducting ballast exchange (MacIsaac et al. 2002b).

Inbound NOBOB vessels could serve as a vector of NIS when they load and then discharge ballast water during 'multi-port' operations on the Great Lakes. Typically, 'multi-port' ships will load ballast at the penultimate ports of call to compensate for discharged cargo. At the last port-of-call, the Great Lakes ballast water — now mixed with residual ballast — is discharged as the outbound cargo is loaded (Fig. 1.2). The addition and subsequent release of water in the Great Lakes, therefore, poses an invasion risk because it provides a direct vector for discharge of live organisms or viable dormant stages of NIS. During this sequence of events, NIS present in residual ballast sediments as dormant stages could be introduced by one of two methods. First, diapausing eggs (i.e., fertilized embryos enclosed in a protective case that lie in a dormant state) may be resuspended from sediments after the addition of freshwater to ballast tanks, such that they are available in the water column for direct discharge to the Great Lakes (Locke et al. 1993). Alternatively, if diapausing eggs were stimulated to hatch inside ballast tanks after loading Great Lakes ballast water, live organisms might be expelled during subsequent deballasting on the upper lakes.

While it has been postulated that NOBOB ships may be responsible for establishment of NIS since ballast exchange regulations were enacted (Locke et al. 1993; Colautti et al. 2003), and that dormant stages in NOBOB sediments could be involved in invasions (Carlton 1985; MacIsaac et al. 2001, 2002b), the magnitude of risk associated with this vector has not been explored. Most previous ballast research has focused on metazoans and microorganisms resident in marine ballast water (Williams et al. 1988; Ruiz et al. 2000; Drake et

al. 2001). Far less attention has been given to ballast sediments and the resting stages therein, particularly in a freshwater context. However, Hallegraeff and Bolch (1991) were able to successfully germinate 20 dinoflagellate species from dormant cysts carried in residual sediment, while Kelly (1993) found that ballast sediments contained viable spores and cysts of diatoms, euglenoid flagellates, ciliates and dinoflagellates. While numerous stakeholders in the Great Lakes region have been calling for proactive legislation on transoceanic vessels, adopting a 'guilty until proven innocent' approach (e.g., Mack et al. 2000), the occurrence of NIS in NOBOB sediments, and the associated propagule pressure, must be quantitatively examined before predictive models can be developed and management protocol soundly determined.

This dissertation describes the first comprehensive examination of the invasion risk posed by invertebrate resting stages in residual sediments of NOBOB vessels. This study characterizes the density, diversity and viability of resting stages transported in residual sediments of transoceanic vessels operating on the Great Lakes and models the introduction potential of NIS to the system using both laboratory and *in situ* hatching experiments.

Chapter II provides the first experimental evidence that diapausing eggs of invertebrate species are present and remain viable after transportation in ballast tanks of transoceanic ships. Diapausing eggs were isolated from residual sediments collected from nine vessels using a sugar flotation method. The viability of diapausing eggs of six zooplankton species was quantified in the laboratory using light:dark cycles of 16:8 and 0:24 to determine optimum hatch

rates under natural day length and ballast tank conditions, respectively. In this manner, I could determine whether or not eggs would hatch if sediments were to be deballasted directly into the Great Lakes, or if the introduction of Great Lakes' water into dark ballast tanks could stimulate hatching *in situ.*

In chapter III, the density and diversity of invertebrate resting stages is characterized for sediments collected from 39 NOBOB vessels. Two types of laboratory studies were conducted to measure hatching abundance and species richness under ideal and more realistic conditions. Data collected from both hatching studies were used to construct a heuristic model of the potential propagule pool transported by NOBOB vessels operating on the Great Lakes. Hatching results are also analysed according to ship ballast history parameters, including location of ballast uptake, ballast capacity and prevalence of saltwater flushing, to determine if risk was related to each ship's activities.

In Chapter IV, the introduction potential of taxa present as resting stages is quantified using *in situ* hatching experiments. Residual ballast sediments were loaded into emergence traps and placed in ballast tanks of four NOBOB ships on their upbound voyage in the Great Lakes. The vessels continued normal operation, loading Great Lakes water into ballast tanks until termination of the experiments at the outbound port-of-call where ballast was discharged. After tanks were emptied, emergence traps were recovered and analyzed for presence of hatching. This is the world's first *in situ* study of hatching of resting stages in ballast tanks of operational ships. These experiments provide the most realistic test of resting egg hatching ability in ships' ballast tanks.

Chapter V explores the salinity tolerance of diapausing eggs of freshwater zooplankton as a potential management strategy. Diapausing eggs of three species, *Bosmina liederi*, *Daphnia longiremis* and *Brachionus calyciflorus*, were collected from residual sediments and exposed to a range of salinities (0, 8, 16 or 32‰) for 10 days. After salinity exposure, diapausing eggs were returned to freshwater media so that hatching rates could be monitored. The effect of the different salinity treatments is described and the utility of salinity as a management tool is discussed.

Finally, Chapter VI briefly synthesizes the novel contributions made by this study and discusses the importance of resting stages in residual ballast sediments in relation to other shipping vectors operating on the Great Lakes.

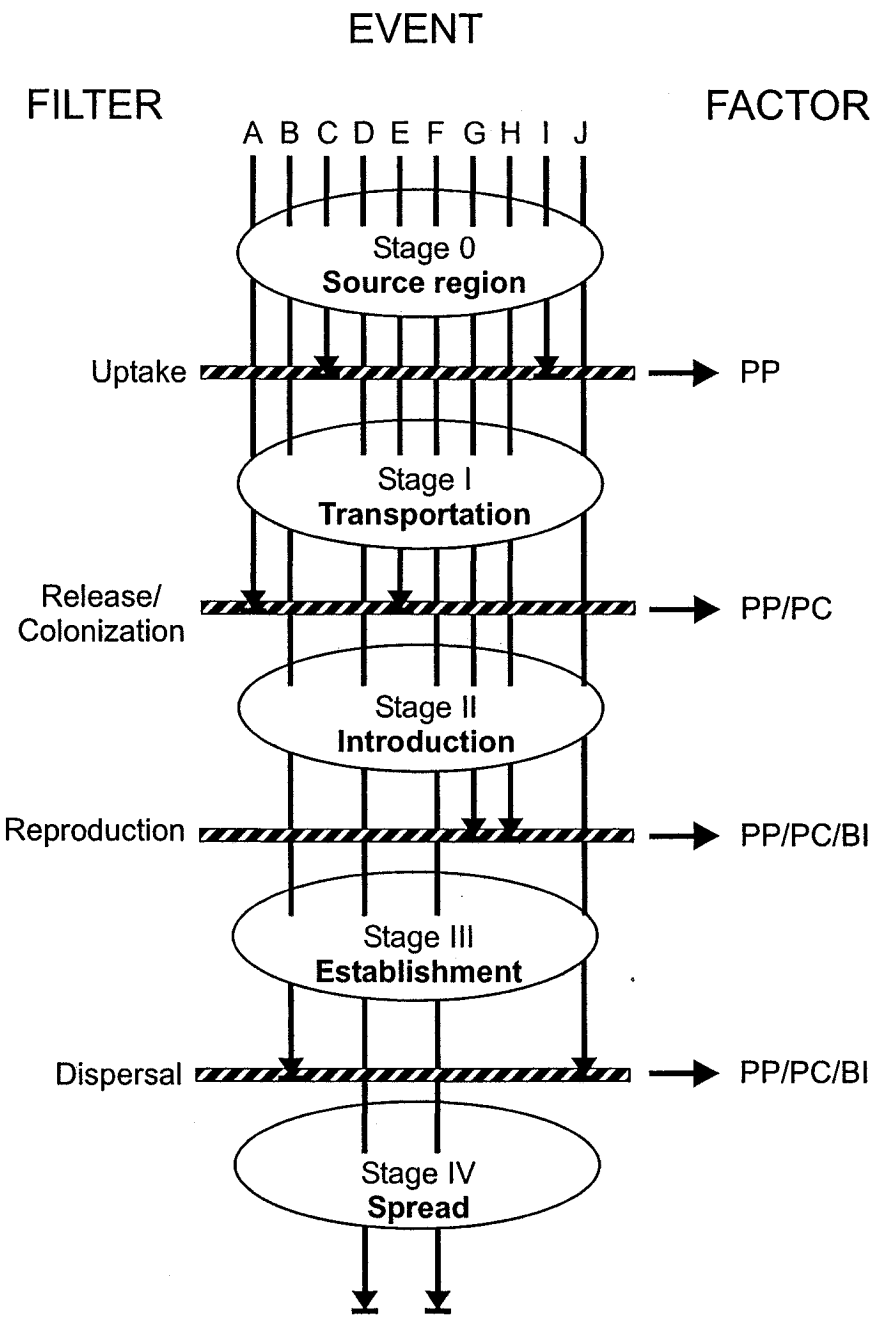

Figure 1.1 Schematic diagram depicting sequence of invasion events, or stages. Potential invaders, A through J, begin as propagules in a source region (stage 0), and pass through a series of filters that may preclude transition to subsequent stages. Factors affecting stage transition are: (PP) propagule pressure; (PC) physico-chemical requirements of potential invader; and (BI) biotic or community interactions. Propagules A through J may represent separate species or conspecific individuals. Adapted from Rahel (2002) and Colautti and MacIsaac (2004).

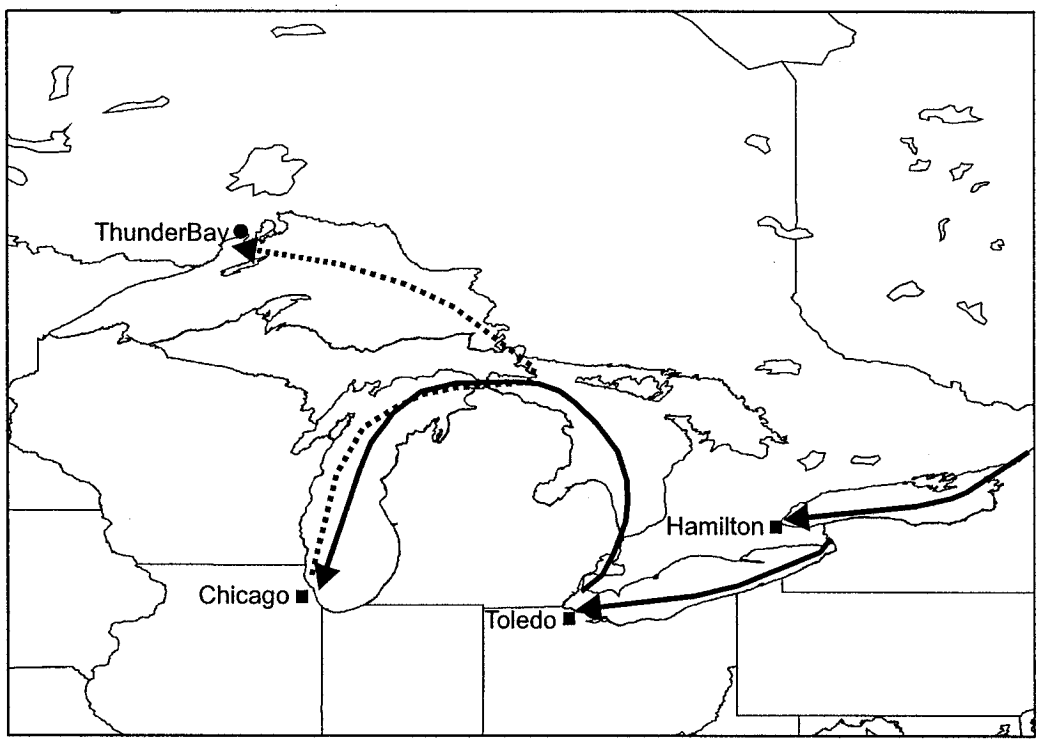

Figure 1.2 Map illustrating typical transit of 'no ballast on board' vessels on the Great Lakes. Vessels enter the Great Lakes fully loaded with cargo and carry only residual ballast. Great Lakes ballast water is loaded to compensate for discharged cargo at several ports (squares). When all cargo is unloaded the vessel carries only "ballast on board" (dotted line). Most vessels visit a final port to load cargo for an outbound destination, typically on Lakes Michigan or Superior; Great Lakes ballast - and any residual water, sediment and/or taxa therein - is discharged to accommodate for the cargo (circle).

VIABILITY OF INVERTEBRATE DIAPAUSING EGGS COLLECTED FROM RESIDUAL BALLAST SEDIMENT

2.1 ABSTRACT

Natural or anthropogenic movement of sediments are an important vector for the dispersal of invertebrate resting stages between water bodies. Here I record the presence of invertebrate diapausing eggs in residual sediments from transoceanic vessels and explore whether these pose a risk of invasion. Viability of diapausing eggs was explored under light and dark conditions using sediment collected from eleven tanks on nine vessels operating on the Great Lakes. Seventeen cladoceran, copepod and rotifer taxa were identified. Four of the species hatched have not yet been reported as established in the Great Lakes. Egg viability for individual species varied from 0 to 92%. Exposure to saline water may impact egg viability of some freshwater species. Generally, the proportion of eggs hatched in light and dark treatments did not differ significantly, indicating that light was not required to terminate diapause. As a result, eggs could potentially hatch in dark ballast tanks when immersed in freshwater loaded as ballast during operation on the Great Lakes. Viability of diapausing eggs differed among ballast tanks on a single vessel, indicating that tanks with independent ballast histories have different invasion risks. While additional work is needed to quantify risk, results from this study indicate that vessels entering the Great Lakes with only residual ballast are a potential vector for the introduction of new nonindigenous species during multi-port operations.

2.2 INTRODUCTION

Freshwater invertebrates achieve dispersal via transport of their desiccation-resistant dormant stages in flowing waters, wind or by ectozoochorous or endozoochorous animal vectors (see Bilton et al. 2001; Cáceres and Soluk 2002; Figuerola and Green 2002). Water currents are likely responsible for most short-distance dispersal events, particularly of stream-dwelling taxa (Bilton et al. 2001). Wind and animal vectors also disperse invertebrates (Figuerola and Green 2002), although recent work indicates that these vectors are species-specific or relatively unimportant (Jenkins and Underwood 1998; Cáceres and Soluk 2002). Movement of sediment represents an alternative dispersal medium that could transport resting stages of many taxa. As an example, sediment associated with heavy machinery has been linked to dispersal of both rotifer and copepod species (Koste and Shiel 1989, Hairston et al. 1999).

Hebert and Cristescu (2002) estimated that the rate of human-mediated dispersal of European crustacean zooplankton to the Laurentian Great Lakes exceeds the natural rate by up to 50,000 fold. Transoceanic ships have been the dominant transport vector of nonindigenous species (NIS) to the Great Lakes for most of the 20th century (Mills et al. 1993; Ricciardi and MacIsaac 2000; Ricciardi 2001). Regulations enacted in 1993 effectively require open-ocean ballast exchange for vessels inbound to the Great Lakes with fresh- or brackish-water if the water is to be discharged into the lakes (USCG 1993). Open-ocean exchange purges most freshwater organisms, while remaining individuals should be killed

when tanks are refilled with saline water. The invasion risk posed by these vessels appears to be much lower than that posed by ships entering without ballast water (MacIsaac et al. 2002b). The latter vessels, officially classified as 'no ballast on board' (NOBOB), are exempt from current ballast water regulations (USCG 1993).

NOBOB vessels, which dominate trade into the Great Lakes, cannot completely empty their ballast tanks due to structural and operational limitations and carry an average of 60 tonnes of unpumpable water and sediments (Colautti et al. 2003; P.T. Jenkins, unpubl.). MacIsaac et al. (2002b) estimated that residual water in NOBOB vessels could carry up to 10^6 zooplankton. Inbound NOBOB vessels could serve as a vector of NIS if they load and then discharge ballast water during multi-port operations on the Great Lakes. Typically, 'multi-port' ships load ballast water when circumstances dictate (e.g., when offloading cargo) to balance longitudinal stresses and to maintain directional stability. At the last port-of-call on the Great Lakes, the residual ballast — now mixed with Great Lakes ballast water — is discharged as outbound cargo is loaded. The addition and subsequent release of water in the Great Lakes, therefore, poses an invasion risk because it provides a direct vector for discharge of live organisms or viable resting stages of NIS. Approximately 51% of NOBOB vessels discharge such mixed ballast water into the Great Lakes system each year (Colautti et al. 2003).

Most previous ballast research has focused on metazoans and microorganisms resident in ballast water (Williams et al. 1988; Ruiz et al. 2000;

Drake et al. 2001). Far less attention has been given to ballast sediments and the resting stages therein. However, Hallegraeff and Bolch (1991) were able to successfully germinate 20 marine dinoflagellate species from dormant cysts carried in residual sediment, while Kelly (1993) found that ballast sediments contained viable spores and cysts of marine diatoms, euglenoid flagellates, ciliates and dinoflagellates.

There exist at least two mechanisms by which invertebrate NIS may be introduced to the Great Lakes via diapausing eggs contained within residual sediments of NOBOB vessels. First, diapausing eggs (i.e., fertilized embryos enclosed in a protective case that lie in a dormant state) may be resuspended from sediments after the addition of freshwater to ballast tanks, such that they are available in the water column for direct discharge to the Great Lakes (Locke et al. 1993). Alternatively, if diapausing eggs were stimulated to hatch inside ballast tanks after loading Great Lakes ballast water, live organisms might be expelled during subsequent deballasting on the upper lakes. In this study, I assess the viability of diapausing eggs in laboratory experiments and test the hypothesis that light is required as a hatching stimulus for diapausing eggs collected from ballast sediments.

2.3 METHODS

Sample collection - Residual sediments were collected from transoceanic vessels entering the Great Lakes in December 2000 and between May and December 2001, at the ports of Hamilton and Thorold, Ontario, Canada, and

Cleveland, Ohio, U.S.A. Vessels were selected opportunistically without regard to previous ports-of-call (i.e., probable sources of ballast water). Residual sediment was collected from at least one tank per ship, and additional tanks were sampled depending upon the time period available and the ease and safety with which the tanks could be accessed. Samples were collected from at least five areas within each tank using sterile scoops and spatulas. Sediments were generally collected along longitudinal shell frames that trapped sediment in areas away from drainage flows. Approximately 4 kg of sediment from a single tank, if available, was composited and later used for experimentation. Temperature and salinity of residual ballast water, if any was present, was recorded at the time of sampling using a Fisher Scientific Traceable® thermometer and a portable optical refractometer. Salinity of sediment pore water was subsequently determined in the laboratory.

Egg Density Counts — Four 40 g subsamples (wet weight) were taken from each sample (except ship 1) and preserved in 95% ethanol. Each subsample was washed through a 45 μm sieve to remove fine sediment. Eggs were subsequently separated from the remaining sediment using a Ludox® HS 40 protocol after Burgess (2001). Mean density of invertebrate diapausing eggs was calculated from the four subsamples after enumeration under a dissecting microscope. Egg densities for ship 1 were estimated by size-fractionating a 130 g sediment subsample from each tank using 1 mm, 500 μm and 250 μm sieves. Each size fraction was examined under a dissecting microscope and total egg

density was calculated per gram of whole sediment. Egg densities from ship 1 were extrapolated to 40 g to compare with other tanks.

Viability Experiments — Unprocessed sediments were stored in plastic containers in the dark at 4°C for at least four weeks to allow a refractory period before hatching experiments commenced (see Grice and Marcus 1981; Schwartz and Hebert 1987). After this time, diapausing eggs were isolated from sediment using the sugar flotation method, modified after Hairston et al. (1995). Sediment was processed through a 45 μm sieve and the retained material washed into centrifuge tubes using a 1:1 mixture (w:v) of sucrose and water. This material was centrifuged at approximately 27 G (~7.7 $m \cdot s^{-2}$) for 5 min. The supernatant was then decanted and rinsed thoroughly with water through a 45 μm mesh before being transferred to a counting dish. Diapausing eggs were immediately recovered from the supernatant and sorted by size and gross morphology under a dissecting microscope, selecting only fully intact, apparently healthy eggs. Eggs were then separated into replicates, with 10 or 20 eggs of one type in each (depending on available quantity), and placed into vials containing 15 ml of sterile synthetic pond water (Hebert and Crease 1980). At least three replicates, depending on egg quantity, were placed into each of two treatments consisting of a light:dark cycle of 16:8 or of 0:24 hours, at 20°C. Exposure to light was minimized during the separation procedure by quickly placing eggs of the dark treatment into vials wrapped in aluminium foil, although some exposure to light occurred during sieving and at the microscope. A sufficient number of eggs for replicated experiments was collected from samples obtained from seven ships

(Table 2.1). Sediment from one double-bottom tank contained only a sparse population of *Bosmina* eggs, thus the number of eggs per replicate in this experiment varied from 4 to 20, although the same number of eggs was used in each light and dark treatment.

A total of twelve distinct egg types were distinguished from residual sediments (Fig. 2.1). Diapausing eggs could be reliably sorted at the genus level, resulting in a total of seventeen taxa emerging from the twelve egg morphotypes. In addition, the hatching of unidentified *Diaphanosoma* sp., *Daphnia* sp. and calanoid copepods may represent additional species, but these organisms were inadequately developed for identification with greater resolution. Only seven of the twelve egg types were found in sufficient density for fully replicated quantitative experiments (see Table 2.1). However, when egg densities were insufficient, eggs were still incubated for identification purposes. Eggs of two species, *Brachionus calyciflorus* and *Bosmina liederi*, were fully replicated from numerous tanks and were used in four and two trials, respectively. Therefore a total of twelve replicated trials, using seven egg types, was executed. Six trials were run for rotifer species, five for cladoceran taxa and one for a calanoid copepod (Table 2.1). Controls containing synthetic pond water were kept in each treatment group to monitor for introduction of organisms from the environment. Vials were checked for emergence every 24 hours for twenty days. Light exposure for eggs in the dark treatment was kept to a minimum, being generally less than thirty seconds per day. Water was renewed every five days and eggs examined for fungal infection or degeneration. Eggs that were contaminated with

fungus were discarded. Few cultures (<1%) had to be discarded for this reason. The number of hatched individuals was recorded daily. Live individuals were removed to separate vials and fed ~12,000 cells·ml^{-1} *Cryptomonas* sp. regularly (University of Toronto Culture Collection #338). All hatched animals were raised to adulthood, when possible, to aid in identification. Taxa were identified using standard taxonomic keys; because the geographical origin of hatched individuals was unknown, at least two keys were utilized. Immature *Daphnia magna* were identified using a mitochondrial cytochrome oxidase subunit 1 assay (P.D.N. Hebert, University of Guelph, pers. comm.). Throughout the duration of the study, all waste, filtered sediments and unhatched eggs were autoclaved prior to disposal to minimize the possibility of environmental contamination.

Variation in the cumulative proportion of diapausing eggs hatched between light and dark treatments was analyzed using a one-way ANOVA with repeated measures using Systat 7.0 (SPSS Inc. 1997). The proportions of eggs hatched were normalized using an arcsine square root transformation prior to analysis. Analyses were conducted only using days when hatching occurred in at least one of the replicates.

2.4 RESULTS

Viability experiments on invertebrate diapausing eggs were conducted on ballast sediment samples retrieved from nine vessels. Salinity of residual water in ballast tanks varied from 0‰ to 46‰, while that of ballast sediment pore water was only slightly less variable (i.e., 0‰ to 37‰) (Table 2.1). Forepeak and

double-bottom tanks from the same ship had disparate ballasting histories. In general, forepeak tanks had not been flushed with saline water, as evidenced by the low salinity of their residual ballast water. For example, the respective salinity of residual water in forepeak and double-bottom tanks for ship 3 was 2‰ and 22‰, while those in ship 4 were 5‰ and 23‰ (Table 2.1).

Species' resting egg densities were generally quite low (i.e., <10 eggs per 40 g sediment) (Table 2.2). There were, however, four instances in which egg density was quite high (> 50 eggs per 40 g sediment) for *Daphnia* spp., *Bosmina* spp., *Brachionus budapestinensis* and *B. calyciflorus*. Cladoceran ephippia were encountered most commonly in ballast sediment, though often at the lowest density. By contrast, diapausing eggs were recovered for fewer rotifer taxa, but they were typically found at much higher densities.

Rotifers (*Asplanchna girodi, Brachionus budapestinensis* and *B. calyciflorus*) generally began to hatch within 24 h of incubation, while calanoid copepods and cladocerans (*Bosmina liederi* and *Daphnia longiremis*) began to hatch at day 3. The average proportion of eggs hatched ranged from 0 to 92%, and was slightly higher in light (40%) than in dark (33%) treatments (Fig. 2.2). Rotifer diapausing eggs exhibited the greatest hatch rate at 92% and averaged 49% over all trials. Typically less than 20% of cladoceran eggs hatched, although in one trial *Bosmina liederi* averaged a 72% hatch rate. Aside from the unusually high viability of *B. liederi* diapausing eggs obtained from it, this tank had no unusual attributes of which I am aware. The copepod trial achieved an 11% hatch rate. Both the number of species present and the viability of eggs varied

among ships and between tanks on a single vessel (see Table 2.1 and Fig. 2.2). No strong relationship between hatch rate and pore water salinity was evident (Fig. 2.3), although high (>65%) hatching success was achieved only for diapausing eggs derived from tanks with low salinity.

A significant difference (ANOVA, $p<0.05$) was observed between light and dark treatments in two trials (Table 2.3; Fig. 2.2D,I); in both instances, hatch rate in the light treatment exceeded that in the dark. However, this pattern was not repeated for other taxa, nor for all the trials combined (Table 2.3). Some diapausing eggs hatched in both light and dark treatments for all but two trials, both of which involved *Bosmina* species. One of these trials had no individuals hatch in either treatment. Two *Brachionus* trials exhibited significant divergence of hatch rates through time, with the light treatment having a higher, steeper curve in both cases (Table 2.3; Fig. 2.2B, D). Overall, there was a very high degree of correspondence between the percent of diapausing eggs that hatched in the light and dark treatments (Pearson correlation 0.93; Fig. 2.4).

2.5 DISCUSSION

This is the first study to experimentally demonstrate that diapausing eggs of invertebrate species are present and remain viable after transportation in ballast tanks of transoceanic ships. Dormancy is a commonly encountered life history strategy in aquatic species that ensures long-term survival through adverse conditions (see Bilton et al. 2001). Resting stages, which can survive periods of anoxia, desiccation and fluctuating temperature (Dodson and Frey 2001; Wallace

and Snell 2001; Lutz et al. 1992), are also essential for dispersal. Colonization success is likely related to the number of dispersal events and the number of propagules in each event. Considering the density of diapausing eggs in residual sediment, and the number of transoceanic vessels transiting the Great Lakes, the dispersal of diapausing eggs via NOBOB vessels is an important invasion mechanism.

Jenkins and Buikema (1998) and Shurin (2000) reported that zooplankton community composition appears to be dispersal-limited. Shipping activities could clearly effect dispersal of species or subspecies within genera that typically have restricted natural distributions. For example, although the genus *Brachionus* is considered cosmopolitan, *B. diversicornis* — a species encountered in one of these ballast sediment samples — has not been reported from either North or South America (Dumont 1983). Likewise, most *Daphnia* species occur in only two or three of the world's six zoogeographical zones (Hebert 1978), and could potentially experience range extensions mediated by shipping activities. In point of fact, an analysis of the cytochrome oxidase 1 gene revealed that the *Daphnia magna* individuals hatched in this study originated from a European, not North American, source (P.D.N. Hebert, pers. comm.). If residual ballast sediments are important dispersal vectors for invertebrates, then the Great Lakes remain vulnerable to invasion despite legal safeguards that prevent the discharge of untreated, freshwater ballast.

In order to estimate the risk of invasion posed by diapausing eggs in residual ballast sediments, it is necessary to consider both their density and

viability. Maximum densities of diapausing eggs in natural populations typically range from 2.0×10^3 to 3.0×10^6 eggs·m^{-2} (Hairston 1996). Assuming that 1 g of sediment is equivalent to 1 cm^3, which seems reasonable considering the high water content of ballast sediment, the densities of diapausing eggs observed during this study (range of 5.0×10^2 to 4.3×10^5 eggs·m^{-2}) are typically an order of magnitude lower than densities observed in natural environments. However, two of the nine vessels had egg densities (5.1×10^4 and 4.3×10^5 eggs·m^{-2}) similar to those reported from ponds, lakes and near-shore marine sediments. In my experience, sediment generally makes up less than 25% of unpumpable ballast. Based on the range of egg densities found in this study, a NOBOB vessel with 15 tonnes of residual sediment could carry 7.5×10^5 to 6.4×10^8 diapausing eggs. With an average hatch rate of 33% in the dark treatment, this translates to approximately 2.5×10^5 to 2.1×10^8 viable propagules per vessel.

The diversity of plankton potentially dispersed by diapausing eggs in ships' residual ballast sediment is quite high, as this study revealed that diapausing eggs of rotifers, cladocerans and calanoid copepods all proved viable after recovery from ballast sediments. However, diapausing eggs of cladocerans and rotifers were much more abundant in ballast sediment than were those of calanoid copepods. In particular, diapausing eggs of the genera *Brachionus* and *Daphnia* were most commonly encountered in residual sediments, with each being recorded from six tanks, although *Brachionus* eggs were much more abundant in ballast sediments than the latter genus. I observed significant differences in hatching success between light and dark treatments in only two of

the twelve trials. This indicates that invertebrate diapausing eggs are generally as likely to hatch in the dark confines of ballast tanks after the intake of freshwater ballast as they are if expelled into a lighted, lake environment. It is interesting and potentially ecologically important that diapausing eggs can be experimentally induced to hatch in the dark as day length has commonly been identified as an important stimulus for hatching of invertebrate diapausing eggs (e.g., Dodson and Frey 2001; Wallace and Snell 2001; but see Arnott and Yan 2002). However, prolonged storage in constant dark conditions has been suggested to eliminate the requirement of light to induce hatching (Stross 1966), and numerous studies have indicated that emergence cues are species- and even genotype-specific (e.g., Schwartz and Hebert 1987; De Meester and De Jager 1993; Arnott and Yan 2002). Diapause eggs may be entrained in ballast sediments, without light stimuli, for weeks, months or years. I propose that the isolation of diapause eggs from ballast sediments, and subsequent exposure to oxygenated media, combined with transfer from cold storage (4°C) to warm incubation (20°C), is sufficient to terminate diapause without exposure to light. Alternatively, it is possible that even brief exposure to lighted conditions, as occurred during examination procedures, was sufficient to initiate emergence, although this seems implausible.

The dense nature of residual ballast sediments is likely to cause the diapausing eggs to be entrapped when loaded with sediment, suggesting that there exists only a small chance that these eggs will be resuspended and expelled with ballast water. Rather, it is more plausible that planktonic individuals

stimulated to hatch from diapausing eggs inside the tanks will be readily available for discharge. It is possible that only those eggs present in surficial sediments will be stimulated to hatch within ballast tanks, as some invertebrate diapausing eggs do not hatch while buried (e.g., Marcus 1996). Therefore, the large number of viable eggs above may be an overestimation of the risk presented unless they are released from sediments during turbulent ballast operations, or are discharged into the Great Lakes directly. Ultimately, *in situ* studies are required to determine if viability from laboratory experiments translates into real risk for vessels operating on the Great Lakes.

The majority of diapausing eggs were hatched during the first ten, and often four, days of the experiments. This pattern is consistent with results from other studies. For example, Schwartz and Hebert (1987) found that *Daphnia* eggs begin to hatch within three to five days when exposed to optimal conditions, while May (1987) showed that the majority of rotifer resting eggs hatched by day ten, and Madhupratap et al. (1996) reported that eggs of four copepod species hatched within 2 days. Most transoceanic NOBOB vessels spend between seven and fourteen days during their inbound voyage on the Great Lakes, during which time they discharge cargo and load ballast water at a series of ports. This interval appears sufficient for diapausing eggs to hatch within tanks, potentially resulting in the production of zooplankton available for discharge with ballast water at the terminal port-of-call.

Of the seventeen taxa that were hatched in this study, four have not been reported as established in the Great Lakes. Two of these species, *Moina affinis*

and *Asplanchna girodi,* occur in the southern United States and/or Mexico (Edmondson 1966; Stemberger 1979; Hebert 1995). It is probable that the eggs of these species were produced by adults, or entered the tanks directly, in ballast loaded at a southern port. For example, *M. affinis* was hatched from a vessel that had recently ballasted at Brownsville, Texas and Veracruz, Mexico. The ship containing diapausing eggs of the European strain of *Daphnia magna* had recently carried ballast from several European countries (Portugal, Germany, Holland and Finland). The fourth species, *Brachionus diversicornis*, has been reported from Australia, Europe, Asia and Africa, but not from either North or South America save for a misidentification of *B. havanaënsis* in the Great Lakes (Stemberger 1979; Dumont 1983). The vessel carrying this species had recently ballasted in Poland (Baltic Sea) and Algeria (Mediterranean Sea). I therefore postulate that Europe or Africa is the likely source for this NIS.

The diversity and viability of diapausing eggs collected from ballast sediments presented here should be considered a minimum estimate. This study was limited by my ability to find and to recognize eggs, and these experiments were designed to assess viability of taxa capable of growing in fresh water at 20°C. I detected no difference in appearance between eggs that did and did not hatch, although it is possible that unhatched eggs were viable but required either longer incubation periods, or different refractory (i.e., pre-treatment) or 'hatch-out' conditions. Conditions required to induce hatching are complex and vary among taxa, although temperature during the refractory and hatching incubation periods are both known to impact the rate of hatching (Schwartz and Hebert 1987). While

20°C will induce hatching of temperate taxa, which are currently of greatest concern to the Great Lakes, arctic or tropical species may perform poorly under these conditions. For example, in one case I observed a hatch rate of only 11% for calanoid copepod eggs at 20°C, while 67-96% of freshwater copepods from natural sediments have been reported to emerge at 8-10°C (De Stasio 1989; Parker et al. 1996). These differences are possibly due to different evolutionary histories of these species and thus may not reflect true egg viability. Furthermore, if eggs have not passed through the necessary refractory period, then even viable diapausing eggs will not hatch under optimal conditions (Grice and Marcus 1981).

Viability of diapausing eggs was highly variable across ships and between the forepeak and double-bottom tanks within a single vessel (Fig. 2.2). These findings are consistent with previous reports of inter-tank variability for dinoflagellates and their cysts in ballast water and sediments, respectively (Hallegraeff 1998; Hamer et al. 2000). This variation may be a consequence of exposure to dissimilar water sources and salinity regimes in different ballast tanks. Forepeak tanks are typically managed differently than double-bottom tanks, and are seldom flushed in mid-ocean. In contrast, it is a common management procedure to flush double-bottom tanks mid-ocean in an attempt to reduce sediment build-up. This procedure also has the added benefit of reducing the number of viable dormant organisms associated with sediments. Differences in operational procedure and tank histories likely result in egg banks of different age, species composition and physiological condition. Consequently, these tanks

would present different risk profiles to recipient systems like the Great Lakes (Hallegraeff 1998; Hamer et al. 2000). Viitasalo and Katajisto (1994) reported that many factors affect the viability of resting stages, such as energy reserves of the eggs, genotype, environmental conditions during egg formation, parental condition, and sediment conditions such as anoxia due to burial. Future research examining the effect of variable salinity conditions on the viability of diapausing eggs with identical histories will be of particular management importance. The salinity tolerances of the taxa involved in this study are previously unexplored. This limited data suggests that exposure to high salinity water may reduce viability of diapausing eggs of freshwater species and potentially reduce the risk posed by NOBOB vessels to the Great Lakes, as the highest hatch rates were observed only for eggs recovered from tanks with relatively low residual ballast water salinity. Yet even at high salinities, a significant fraction of eggs often did hatch. If diapausing eggs of freshwater invertebrates are indeed capable of surviving drastic and abrupt changes in salinity, it could have vast implications for the long-term persistence of these taxa in environments experiencing temporary salinity increases, such as tidal estuaries and in waterbodies subject to evaporation. Finally, the widespread presence of viable diapausing eggs in residual ballast sediments, produced by a diverse group of invertebrate taxa, indicates that commercial NOBOB vessels have the potential to introduce NIS to the Great Lakes.

Table 2.1 List of species hatched from eleven ballast tank sediments through quantitative and qualitative hatching studies. Species with N/A replicates were too sparse to obtain enough eggs for fully replicated experiments and were hatched only for identification purposes. Salinity was measured from residual ballast water in tanks and as pore water extracted from sediment. FP = forepeak tank, DB = double-bottom tank.

Ship	No. of replicates	Species	Salinity	
			Ballast water	Pore water
1(FP)	5	*Daphnia longiremis*	46	37
	N/A	Chydoridae		
2(DB)	N/A	*Moina affinis*	24	34
3(FP)	3	*Bosmina liederi*	2	2
	N/A	*Daphnia* sp.		
	N/A	*Diaphanosoma* sp.		
3(DB)	4	*Bosmina liederi*	22	20
	N/A	*Daphnia ambigua*		
4(FP)	3	*Brachionus calyciflorus*	5	10
	N/A	Calanoid copepod		
	N/A	*Daphnia magna*		
	N/A	*Diaphanosoma brachyurum*		
	N/A	*Filinia* sp.		
	N/A	*Moina micrura*		

Table 2.1 (continued)

4(DB)	3	*Brachionus calyciflorus*	23	18
	N/A	*Brachionus budapestinensis*		
	N/A	*Daphnia magna*		
5(DB)	5	*Asplanchna girodi*	2	0
	5	*Brachionus budapestinensis*		
	N/A	*Brachionus angularis*		
	N/A	*Ceriodaphnia pulchella*		
	N/A	*Moina micrura*		
6(DB)	N/A	*Daphnia* sp.	N/A	35
7(DB)	5	*Bosmina liederi*	0	2
	N/A	*Brachionus calyciflorus*		
	N/A	*Brachionus diversicornis*		
	N/A	*Brachionus budapestinensis*		
	N/A	*Ploesoma* sp.		
8(DB)	5	*Brachionus calyciflorus*	20	19
	N/A	*Brachionus budapestinensis*		
	N/A	*Brachionus angularis*		
	3	*Bosmina* sp. (0 hatched)		
	4	Calanoid copepod		
9(DB)	5	*Brachionus calyciflorus*	37	28

Table 2.2 Average density of diapausing eggs in 40 g subsamples of residual ballast sediments. Eggs classified as rare were not encountered in all four subsamples and had an average density <2 eggs per 40 g. FP = forepeak tank, DB = double-bottom tank.

Ship	Species	No. eggs per 40 g
1(FP)	*Daphnia* spp.	202
	Chydoridae	rare
2(DB)	*Moina* spp.	3
3(FP)	*Bosmina* spp.	56
	Daphnia spp.	3
	Diaphanosoma spp.	rare
3(DB)	*Bosmina* spp.	4
	Daphnia spp.	rare
4(FP)	*Brachionus calyciflorus*	4
	*Brachionus budapestinensis**	4
	Calanoid copepod	rare
	Daphnia spp.	rare
4(DB)	*Brachionus calyciflorus*	7
	Brachionus spp.	37
	Daphnia spp.	rare

Table 2.2 (continued)

5(DB)	*Asplanchna* spp.	14
	Brachionus calyciflorus	51
	*Brachionus budapestinensis**	1703
	Ceriodaphnia spp.	rare
	Moina spp.	rare
6(DB)	*Daphnia* spp.	rare
7(DB)	*Bosmina* spp.	6
	Brachionus calyciflorus	2
	*Brachionus budapestinensis**	rare
	Ploesoma spp.	rare
8(DB)	*Brachionus calyciflorus*	6
	*Brachionus budapestinensis**	17
	Bosmina spp.	rare
	Calanoid copepod	3
9(DB)	*Brachionus calyciflorus*	23

* While 99% of the organisms hatched from these eggs were *Brachionus budapestinensis,* individuals of *B. angularis and B. diversicornis* also hatched from this egg morphotype.

Table 2.3 ANOVA with repeated measures demonstrating the effect of illumination treatment on the hatch rate of diapausing eggs. Data were arcsine square root transformed prior to analysis. Significance levels for *F*-values: NS ($p>0.10$), * ($p<0.05$), ** ($p<0.0001$). FP = forepeak tank, DB = double-bottom tank.

		ANOVA effects *F* value (df)		
Ship	Organism	treatment	time	time*treatment
1(FP)	*Daphnia longiremis*	6.5* (1,8)	16.8** (12,96)	0.6^{NS} (12,96)
3 (FP)	*Bosmina liederi*	0.1^{NS} (1,5)	4.0** (6,30)	0.3^{NS} (6,30)
3 (DB)	*Bosmina liederi*	0.1^{NS} (1,7)	1.8* (20,140)	0.1^{NS} (20,140)
4(FP)	*Brachionus calyciflorus*	0.3^{NS}(1,7)	0.0** (20,140)	0.9^{NS} (20,140)
4(DB)	*Brachionus calyciflorus*	0.1^{NS} (1,4)	0.1^{NS} (3,12)	0.1^{NS} (3,12)
5(DB)	*Asplanchna girodi*	1.7^{NS} (1,8)	4.9** (6,48)	1.7^{NS} (6,48)
	Brachionus budapestinensis	3.3^{NS} (1,8)	1.9** (8,64)	2.5* (8,64)
7(DB)	*Bosmina liederi*	0.2^{NS} (1,8)	15.2** (6,48)	0.9^{NS} (6,48)
8(DB)	*Brachionus calyciflorus*	2.6^{NS} (1,9)	56.0** (3,27)	1.5^{NS} (3,27)
	Bosmina sp.	0.7^{NS} (1,5)	0.5^{NS} (20,100)	0.7^{NS} (20,100)
	Calanoid copepod	0.1^{NS} (1,7)	9.8** (7,49)	0.2^{NS} (7,49)

Table 2.3 (continued)

9(DB)*Brachionus calyciflorus*	15.6* (1,8)	1.4** (9,72)	11.4** (9,72)
All trials combined	0.4^{NS} (1,97)	91.5** (20,1940)	0.3^{NS} (20,1940)

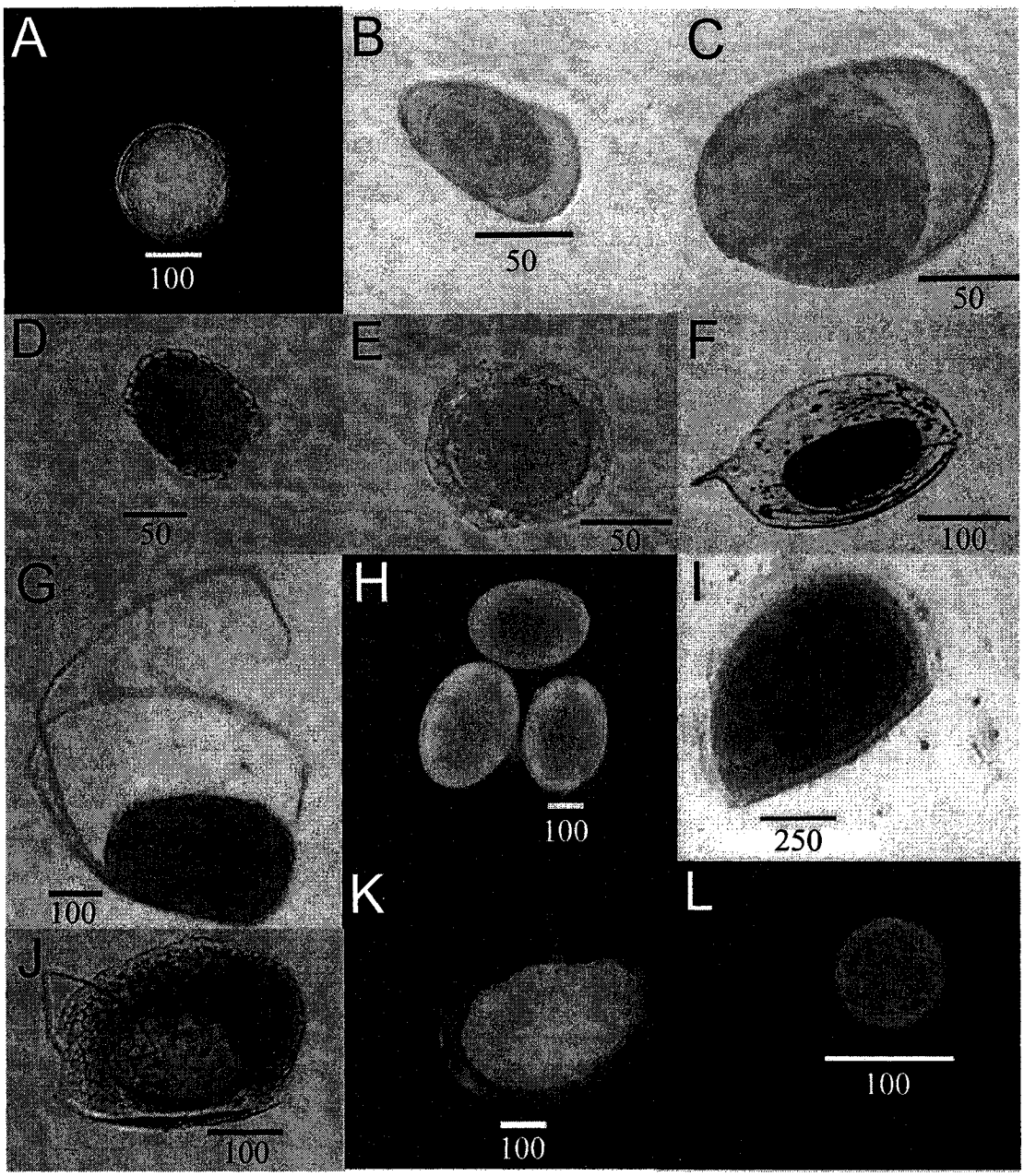

Figure 2.1 Resting egg morphotypes successfully recovered from ballast sediments. Rotifera: (A) *Asplanchna girodi,* (B) *Brachionus budapestinensis,* (C) *B. calyciflorus,* (D) *Filinia* spp., (E) *Ploesoma* spp.; Cladocera: (F) *Bosmina* spp., (G) Chydoridae, (H) various Cladocera, (I) *Daphnia* spp., (J and K) *Moina* spp.; Copepoda: (L) calanoid copepod. Scale bars (μm) are included on each image.

Figure 2.2 Mean (± S.E.) cumulative proportion of diapausing eggs hatched under light and dark treatments, by taxon. (A) *Asplanchna girodi*, (B) *Brachionus budapestinensis*, (C) *Brachionus calyciflorus* (ship 8), (D) *B. calyciflorus* (ship 9), (E) *B. calyciflorus* (ship 4), (F) *Bosmina liederi* (ship 3), (G) *Bosmina liederi* (ship 7), (H) *Bosmina* sp., (I) D*aphnia longiremis*, and (J) calanoid copepod. Circle and square symbols represent double-bottom and forepeak tanks, respectively. Error bars less than 0.04 are hidden by graph symbol. * and ** denote trials for which differences in treatment and time*treatment were significant, respectively. † denotes a significant difference between tanks on a single vessel. All other growth curves differed insignificantly from each other (ANOVA, $p>0.05$).

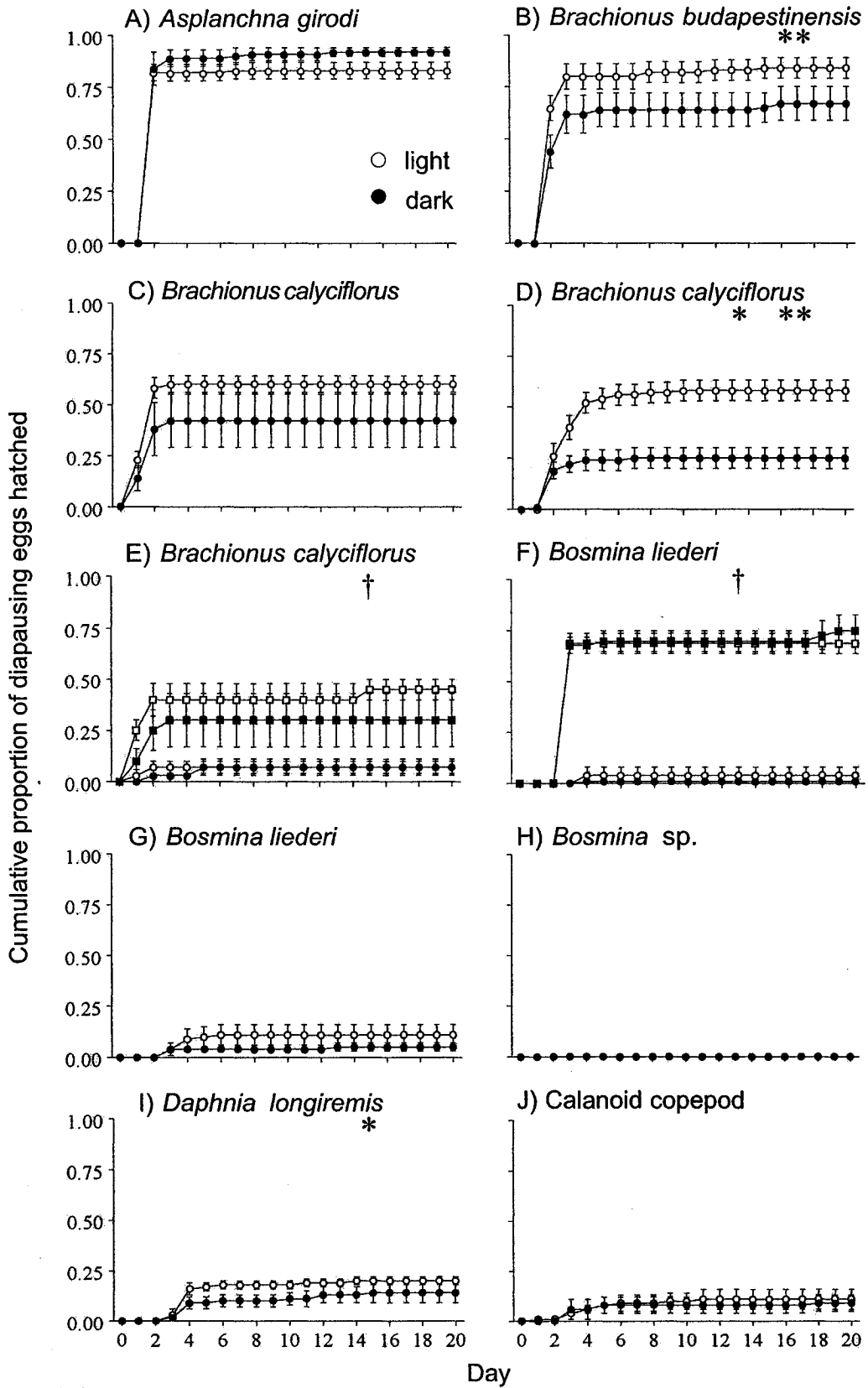

Figure 2.2

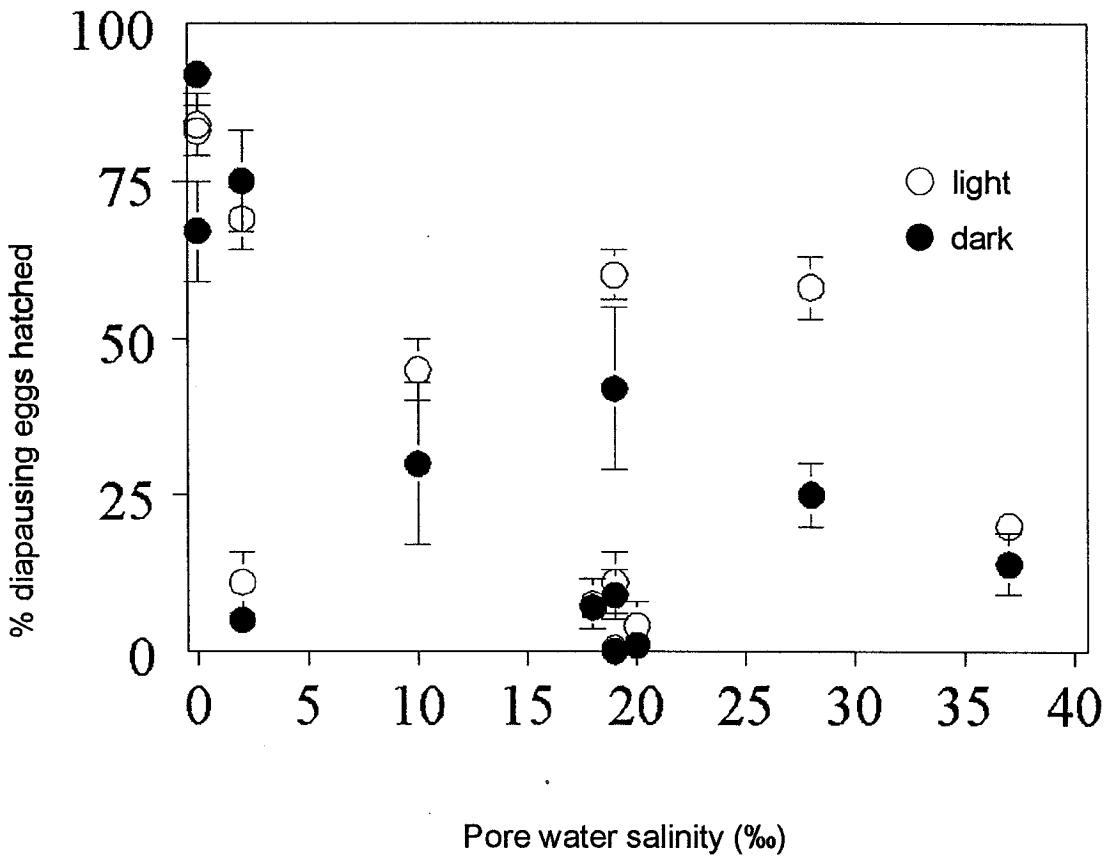

Figure 2.3 Mean (± S.E.) proportion of diapausing eggs hatched as a function of pore water salinity.

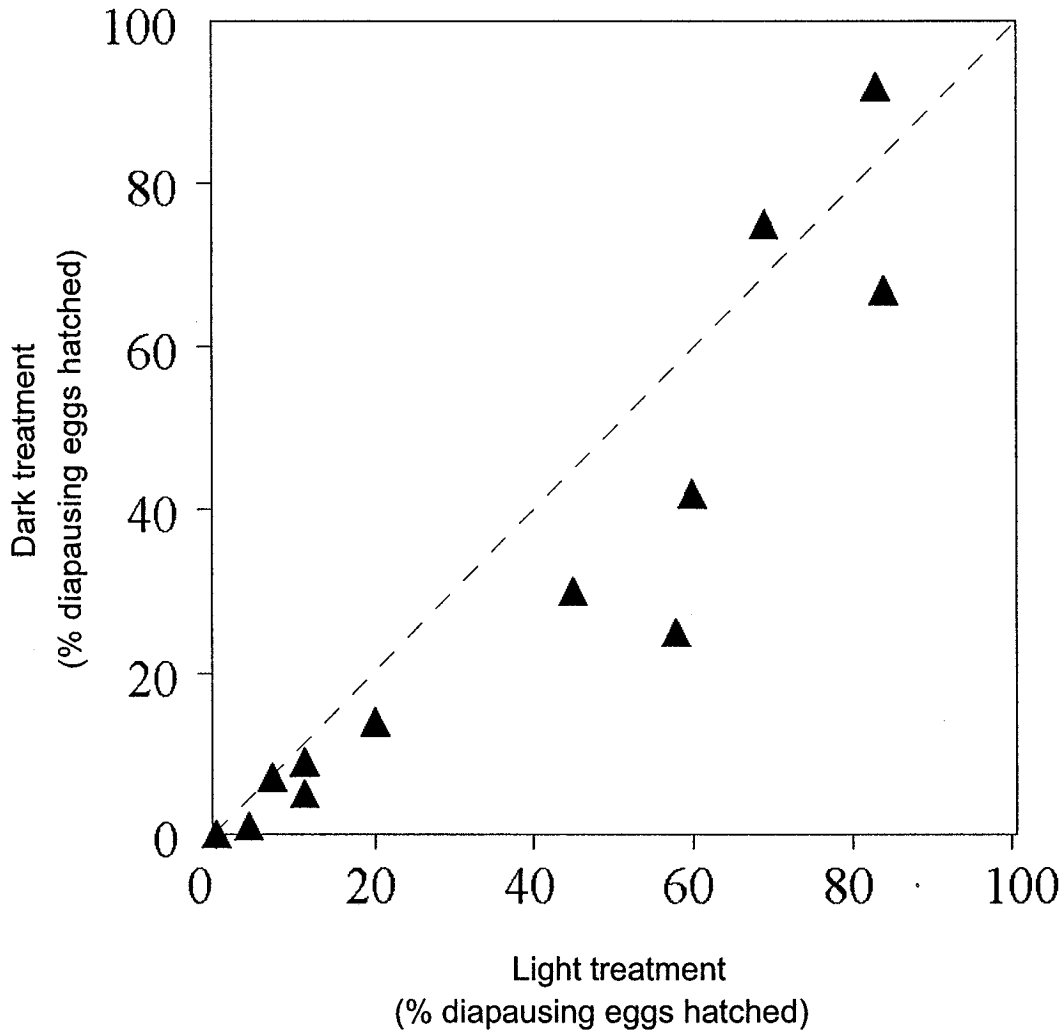

Figure 2.4 Percent diapausing eggs that hatched in the dark relative to that in the light treatment. Each point represents one trial. The Pearson correlation is 0.93.

INVERTEBRATE RESTING STAGES TRANSPORTED IN RESIDUAL BALLAST SEDIMENT OF TRANSOCEANIC SHIPS

3.1 ABSTRACT

Ballast water has been the primary vector of nonindigenous species (NIS) to the Laurentian Great Lakes over the past 45 years. Although ballast water exchange regulations were implemented in 1993 to reduce propagule loads, new NIS continue to be discovered. A possible explanation for this trend is the importance of alternative vectors, such as residual ballast of ships claiming 'no ballast on board'. Here, I investigate resting stages of invertebrates in residual ballast sediments of transoceanic ships as a possible vector of NIS to the Great Lakes. To model the introduction effort potentially associated with this vector, I collected sediment samples from 39 ships entering the Great Lakes and measured the density, viability and species richness of resting stages contained therein. Viable resting stages of NIS were found in 32% of ships, at a median density of 3.0×10^5 ship^{-1}. Temperature, salinity and removal of eggs from sediment during incubation had a significant impact on total abundance and species richness of hatched taxa. Twenty-one NIS were identified in total, consisting exclusively of rotifers and cladocerans. Salinity of residual ballast water and geographic region of ballast uptake were predictive variables for profiling invasion risk of ships, although explained variability was low.

3.2 INTRODUCTION

The introduction of nonindigenous species (NIS) beyond their native ranges is a major threat to global biodiversity, particularly for lake ecosystems (Brönmark and Hansson 2002; Rahel 2002). Because economic and ecological costs associated with NIS are significant (e.g., Pimentel et al. 2000; Colautti et al. 2005b), predicting and preventing invasions is an increasingly important global priority (Kolar and Lodge 2001). Predicting invasion success requires knowledge of all of the stages inherent in an introduction event, including uptake, transportation, release and establishment (Kolar and Lodge 2001; Sakai et al. 2001). The starting point, and arguably the most important aspect of an invasion, is the introduction effort or propagule pressure associated with transfer of NIS to new areas (Ruiz et al. 2000; Kolar and Lodge 2001; Duncan et al. 2003; Colautti and MacIsaac 2004).

Vectors associated with transoceanic shipping (e.g., ballast water, hull fouling) are recognized as the largest source of aquatic NIS introductions globally (e.g., Ruiz et al. 2000; Leppäkoski et al. 2002; Lewis et al. 2003), and account for 67% of the species introduced to the Laurentian Great Lakes since 1959 (Grigorovich et al. 2003a). Changes in the type of NIS associated with shipping have occurred concomitant with a transformation in the nature of ships' ballast. Initial invaders to the Great Lakes were predominantly plants, transported as seeds in solid ballast; this pattern shifted during the 20th century to dominance by algae and invertebrates carried by liquid ballast (Ricciardi and MacIsaac 2000; Colautti et al. 2003). A number of invertebrate species that inhabit the sediment-

water interface have been reported since 1988 (Holeck et al. 2004), possibly reflecting another shift as the relative importance of ships claiming 'no ballast on board' (NOBOB) status increased post ballast water exchange regulations (Colautti et al. 2003).

NOBOB ships are exempt from current ballast water exchange legislation because their ballast tanks are considered empty. However, NOBOB ships carry an average of 60 tonnes of residual water and sediments while operating on the Great Lakes (P.T. Jenkins, unpubl.). NOBOB ships pose a risk of introduction of NIS to the Great Lakes because they theoretically carry more live freshwater individuals and participate in a greater number of inoculation events than do ballasted vessels (MacIsaac et al. 2002b; Colautti et al. 2003). Dormant resting stages in residual sediments are a potentially important contributor to the number of propagules carried by NOBOB ships (Bailey et al. 2003; Chapter II); however this mechanism has not been thoroughly quantified.

Dormant resting stages are produced by many invertebrates, particularly freshwater taxa, and occur in various forms at different life history stages (e.g., diapausing eggs, dormant buds, quiescent juveniles and anhydrobiotic adults; see Cáceres 1997 for a review). While distributions of many rotifer and cladoceran taxa capable of producing resting stages have been considered cosmopolitan owing to widespread passive dispersal, recent work has shown a greater prevalence of endemism between continents (Dodson and Frey 2001; Wallace and Snell 2001). As transfer of resting stages in relocated sediments has been previously implicated as a vector for zooplankton introductions

(Schrimpf and Steinberg 1982; Koste and Shiel 1989; Hairston et al. 1999), and considering that over one hundred million tonnes are being transported internationally by ships annually (Endresen et al. 2003; assuming sediment comprises <10% of ballast water), ballast sediments have emerged as a potentially important source of NIS.

My previous study of diapausing eggs in ballast sediments of ships trading on the Great Lakes was preliminary, examining egg viability of a few dominant species from nine ships (Bailey et al. 2003; Chapter II). Studies of ballast sediments in ships for other trade areas have also been limited, focused primarily on marine dinoflagellates and phytoplankton (e.g., Kelly 1993; Smith et al. 1996; Hamer et al. 2000). In this study, I test the hypothesis that NOBOB ships are a transport vector of NIS to the Great Lakes. I sampled transoceanic ships entering the system during a two-year period to quantitatively characterize the density, viability and richness of dormant propagules carried in residual ballast sediments. I use these results to construct a heuristic model of the propagule pressure associated with sediments of NOBOB ships, and to predict the number of NIS carried by NOBOB ships to the Great Lakes annually. Finally, I test the hypothesis that ballast history parameters can be used to predict which ships present a 'high risk' of causing new invasions.

3.3 METHODS

Thirty-nine transoceanic ships in NOBOB status were boarded for collection of residual sediments from 69 ballast tanks between December 2000 and

December 2002, inclusive. This sample size represents approximately 16% of the annual 'multi-port' NOBOB traffic in the Great Lakes (i.e., NOBOB ships which fill and empty ballast tanks within the Great Lakes). Detailed collection methods are described in Chapter II. Salinity of residual ballast water, if any was present, was measured at the time of sediment collection using an optical refractometer. Information regarding ships' ballast histories and other physical parameters were recorded at the time of sampling (see Analysis of Ballast History). Four ships were sampled twice during the sampling period, with each independent trip into the Great Lakes considered a unique ship sample since new ballast had been held in tanks between sampling periods.

I calculated the Sorensen's coefficient of similarity (Krebs 1999), based on presence/absence of species in each sample, for pairs of tanks within and between ships to analyse both the spatial and temporal variation in community composition. Sorensen's coefficient typically ranges from zero to one, with higher values indicating greater similarity of samples. First, to determine whether tanks from the same ships were biologically more similar than randomly drawn pairs of tanks from different ships, I contrasted Sorensen's coefficients for all pairs of tanks within ships (n=17) against those for 1000 randomly drawn boot-strapped pairs of tanks between ships using a Mann-Whitney *U*-test (Systat 8.0, SPSS Inc., 1998). Secondly, to confirm that the four ships sampled on two occasions should be treated as independent samples, I contrasted Sorensen's coefficients for all pairs of tanks on repeated ships (n=10) against those of the same 1000 randomly drawn boot-strapped pairs of tanks between ships using a Mann-

Whitney *U*-test. Only one of the 10 pairs of tanks was a true, temporally replicated sample, with the same tank sampled at both time periods. The other 'pairs' were independent tanks that had each been sampled once, with one tank sampled during the first visit and a different tank sampled on the second visit. Since the spatial analysis determined that tanks within ships at a single time point are more similar than random pairs, I included these 'pairs' as replicates for investigation of temporal trends. Species lists generated from maximum diversity experiments conducted in 0‰ medium at 20°C (described below) for 47 tanks on 29 ships were used to calculate all Sorensen's coefficients.

Resting Stage Density Counts — After thorough mixing, four 40 g sediment subsamples (wet weight) were taken from each ballast tank sample and preserved in 95% ethanol. Resting stages were enumerated under a dissecting microscope after separation from coarse sediment using the colloidal silica Ludox® HS 40. Average density calculated from the four subsamples was subsequently converted to density of resting stages per tonne of sediment.

Hatching Experiments — Unprocessed sediments were stored in plastic containers in the dark at 4°C for at least four weeks to allow a refractory period before hatching experiments commenced (see Grice and Marcus 1981; Schwartz and Hebert 1987). After this time, sediments were stirred manually, and 40 g subsamples were removed in four 10 g allotments. Synthetic pond water of 0‰ salinity (Hebert and Crease 1980) or serial dilutions (8, 16, or 32‰) of filtered, natural seawater were used as hatching media. Natural seawater for these experiments was collected from a ship loaded with ocean water ballast, and

filtered through a 0.2 μm Whatman paper filter. All experiments were conducted using a light:dark cycle of 16:8 hours. I conducted two types of studies: maximum diversity and whole sediment experiments.

Maximum diversity experiments were designed to promote maximum hatching abundance of the dormant taxa in the sediment community to assess species richness and abundance across ships. Resting stages were separated from sediments collected from five tanks (four ships), selected for high density of resting stages, using a sugar flotation method (Bailey et al. 2003; Chapter II). Briefly, four 40 g subsamples of each tank sediment were processed through a 45 μm sieve to remove fine sediment before being washed into centrifuge tubes using a 1:1 (w:v) mixture of sucrose and water. After centrifugation for 5 min at 27 G (~7.7 $m \cdot s^{-2}$) the supernatant was decanted and rinsed thoroughly with water through 45 μm mesh. The supernatant for each subsample was subsequently transferred to a 9 cm^2 Petri-dish containing 40 ml of sterile medium. Four replicates were incubated in each of four treatments: 0‰ and 8‰ media at each of 10°C and 20°C. Dishes were checked for emergence every 24 hours for the first ten days, and every 48 hours for the subsequent ten days. All hatched individuals were immediately removed for enumeration and identification. Controls containing blank growth medium were kept in each treatment group to monitor for introduction of organisms from the environment. Sediments from an additional two tanks were incubated only in 0‰ medium at 20°C for 10 days. Variation in total abundance and species richness hatched between salinity and temperature treatments was analyzed using two-way MANOVA (Systat 8.0,

SPSS Inc., 1998). The two 10-day experiments were excluded from analyses for consistency. If a significant multivariate effect was observed, univariate ANOVA was performed to discern the effect of salinity and temperature on each dependent variable. Both total abundance and species richness were transformed to improve normality before analysis. As these experiments were extremely labour-intensive, an unreplicated 40 g sediment sample was prepared in the same way, incubated in 0‰ medium at 20°C for 20 days, for all remaining sediments (50 tanks from 28 ships). In this manner I could collect information on richness and abundance of common species from a broad array of ships with reduced overall effort.

Whole sediment experiments were designed to give a more realistic estimate of hatching abundance and species richness *in situ*. The protocol used was modified from that of May (1986) and Duggan et al. (2002). Four 40 g subsamples were removed from each of nineteen tank sediments (sixteen ships) and placed into 500 ml glass vessels. 150 ml of 0‰ medium was added to each vessel before incubation at 20°C. Vessels were swirled by hand to mix the sediment with the medium. Vessels were examined for emergence of invertebrates every 48 hours for 20 days by carefully decanting the medium through a 45 μm mesh screen. All material retained on the mesh was washed into a counting tray for enumeration and identification. Growth medium was immediately returned to each vessel. Vessels were examined at time 0 to be certain that any active copepods present in the sediment would not be later mistaken for those awakening from quiescent stages; one taxon in one

experiment was disregarded for this reason. Controls containing autoclaved sediment were kept in each treatment group to monitor for introduction of organisms from the environment.

Three additional salinity treatments, 8, 16 and 32‰, were added for a subset of the whole sediment experiments (ten tanks from eight ships) to determine if brackish or saltwater taxa were also present in the sediment egg bank. Variation in total abundance and species richness hatched between salinity treatments was analyzed using one-way MANOVA (Systat 8.0, SPSS Inc., 1998). Both total abundance and species richness were transformed to improve normality before analysis. Sediments analyzed for 20 days in both the replicated diversity and whole sediment experiments in 0‰ medium at 20°C were analyzed by one-way MANOVA to determine if total abundance and species richness hatched differed as a result of experimental method (n=5). Again, all significant MANOVA results were subsequently investigated using ANOVA and Bonferoni *post hoc* tests were used to determine differences between the four salinity levels on each dependent variable.

For all experiments conducted in the laboratory, hatched individuals were removed to separate vials and raised to maturity, when possible, to aid in identification. Taxa were identified using standard taxonomic keys. No individuals were recovered from control vials at any time. All waste generated during the experiments was autoclaved prior to disposal to minimize the possibility of environmental contamination.

Analysis of Ballast History — I recorded ballast history information, obtained from each ship's crew, to determine whether risk identified from hatching trials was related to each ship's activities. Information collected included total ballast capacity, previous dates and locations of ballast uptake, and prevalence of saltwater exchange or tank flushing. As some ships did not have records beyond the last change of crew, management and/or ownership, I could only obtain a sufficient set of records for the two most recent ports of ballast uptake. Locations of ballast uptake were assigned to one of 7 broad geographical regions: 'Baltic Sea', 'Great Lakes basin' (including the St. Lawrence Seaway), 'Mediterranean and Black Seas', 'North Sea', 'north-west Pacific Ocean', 'west-central Atlantic Ocean' and 'other'. In addition, current and historical records of tank residuals from ship crews, as well as visual observations made by an experienced shipping vessel consultant (P.T. Jenkins), were used to estimate the amount of residual sediment aboard each ship.

Most tanks on the same ship have identical ballast histories, thus I chose to average data for these tanks to avoid pseudoreplication problems. However, I excluded data collected from six tanks, each from separate ships, because the salinity of the residual ballast water in those tanks could not be explained by the ballast history provided by the crew. Averaging tanks for this truncated dataset was further justified by separate paired *t*-tests of residual water salinity and resting stage density, each of which found no significant difference between paired tanks within ships. Forward- and backward-stepwise multiple regressions were used to determine if any of the continuous ballast history variables (i.e.,

residual water salinity, total ballast capacity, volume of residual sediment and month of last ballast uptake) were important predictor variables of resting stage density or total abundance of hatched invertebrates. Subsequently, ANCOVA was used to investigate the relationships between both egg density, and total abundance hatched, with previous regions of ballast uptake. Again, only total abundances hatched in 0‰ at 20°C during maximum diversity experiments were used for analysis, as this was my most comprehensive dataset. Dependent and independent variables were transformed when necessary to improve normality prior to analysis.

Estimation of Propagule Pressure — I calculated the number of viable dormant propagules, π_{φ}, carried by any ship as:

(1) $$\pi_{\varphi} = \delta\varphi\tau$$

where δ is the density of resting stages per tonne of sediment for that vessel, φ is the proportion of resting stages that are viable and τ is the amount of sediment in tonnes aboard the vessel. I calculated propagule pressure for the 34 ships analyzed by the maximum diversity experiments above, using parameter values generated in 0‰ medium at 20°C. To deduce the number of viable NIS propagules carried, π_{ν}, I added a term, ν, to indicate the proportion of viable propagules that are considered nonindigenous to the receiving area:

(2) $$\pi_{\nu} = \delta\varphi\tau\nu$$

where ν is the product of the number of nonindigenous individuals divided by the total number of individuals. The method of maximum likelihood coupled with parametric bootstrapping techniques (Efron and Tibshirani 1998) was used to

estimate the mean and confidence limits for the number of resting stages of NIS carried by NOBOB ships (X), when present, using a log-likelihood function for an exponential distribution (Eqn. 3), after removal of one outlier point (see Discussion):

(3) $$l(\omega|X) = \sum_{i}^{n} (\ln \omega - \omega * x_i)$$

where ω = 3.39064 x 10^{-6} is the estimate when equation 3 is maximized.

To determine the total number of species associated with NOBOB ships deballasting in the Great Lakes annually, I conducted a Monte Carlo simulation of the cumulative number of non-redundant species (i.e., not identified from a previously selected ship) as a function of the number of ships sampled using data generated from both maximum diversity and whole sediment experiments. I randomly selected samples of incremental size, from 1 to 36 ships, without replacement. This procedure was repeated for 500 bootstrap iterations, with the average and standard error of the cumulative number of non-redundant species calculated. A species-area curve was fitted as an asymptotic (i.e., hyperbolic) curve to the average of the iterations using Statistica 5.5 (Statsoft Inc., 2000), after preliminary inspection of the data. I used least-squares loss functions and Hooke-Jeeves/Quasi-Newton root solving methods (Hooke and Jeeves 1961) to build the non-linear model describing the number of non-redundant species as:

(4) $$y = \frac{\alpha\beta x^{\theta}}{1 + \beta x^{\theta}}$$

where y is the mean cumulative number of non-redundant species, x is the number of ships sampled, and α, β and θ are estimated parameters. Species-

area curves were developed separately for species considered indigenous and nonindigenous to the Great Lakes. Although most concern currently centres on the latter group, it is possible that intraspecific genetic invasions may occur for some species in the former. Finally, I extrapolated the species-area curves to 250 ships, which is typical of the number of 'multi-port' NOBOB ships deballasting in the Great Lakes any given year (Colautti et al. 2003).

3.4 RESULTS

The amount of residual ballast sediment ranged from <1 to 65 tonnes per ship, with an average value of 14.4 tonnes. Spatial variation in community composition of tanks within ships was significantly lower than that of tanks between ships, with mean Sorensen's coefficients of 0.35 and 0.25, respectively (*U*-test, $p<0.05$; Fig. 3.1). In contrast, ships sampled on two occasions showed significant temporal variation in community composition, as Sorensen's coefficients for tanks on repeated ships were not significantly different than those of 1000 randomly-selected pairs of tanks between ships (Mann-Whitney *U*-test, $p>0.05$; see Fig. 3.1).

The density of invertebrate resting stages in ship sediments had a lognormal distribution, ranging from 4.0×10^4 to 9.1×10^7 resting stages·tonne^{-1} (median and mean values of 7.2×10^5 tonne^{-1} and 3.6×10^6 tonne^{-1}, respectively). Taxonomic identity, based upon resting stage morphology, was made for 12 groups in the sediments (see Table 3.1). Clearly, total species richness was underestimated, as many resting stages could not be differentiated

beyond genus, while other, smaller taxa (e.g., Dicranophoridae) may have been overlooked. Diapausing eggs of rotifers, particularly *Brachionus* species, dominated (77.9%) resting stage abundance. This pattern was influenced by one ship with an extremely high density of *Brachionus* diapausing eggs (65.3% of resting stage abundance for all ships); although the same general pattern held true when that ship was excluded.

Sufficient quantities of sediment for laboratory experiments were lacking in three ships, limiting assessments to density of resting stages. For the remaining 36 ships, I hatched 76 distinct taxa. Twenty-one NIS were identified, consisting of 14 rotifers and 7 cladocerans. One NIS identified in this study, *Bosmina maritima*, has already become established in the Great Lakes (Table 3.2). Both the total abundance and frequency of occurrence of NIS was low in comparison to species considered native to the Great Lakes (see Appendix).

In the maximum diversity experiments, 59 taxa were hatched from the replicated trials, although this number is conservative owing to the presence of unidentifiable juvenile invertebrates. Species richness ranged from 0 to 20 taxa per sediment, with a median value of 3. Thirteen additional unique taxa, of 45 in total, were identified from the 50 unreplicated diversity trials. All taxa were hatched from true diapausing stages; no quiescent copepodids were recovered by this method. The rotifer *Synchaeta bacillifera* and the cladoceran *Evadne nordmanni*, were the only organisms that hatched exclusively in brackish water. The Rotifera was the most species-rich group, comprising 75% of all species hatched. Cladocerans comprised the second richest taxon, representing 23% of

hatched species. Copepod nauplii were hatched from 14 sediments, though they could not be identified to the species level and were considered as one taxon in consequence. The 0‰, 20°C treatment group had both the highest abundance and greatest species richness hatched, followed by the 0‰, 10°C treatment group (Fig. 3.2). Both total abundance and species richness were significantly affected by experimental temperature and salinity (MANOVA, $p<0.01$; Table 3.3). Univariate analyses indicated that higher salinity and lower temperature each suppressed total abundance and species richness of hatched taxa independently, and that there was no interaction effect for salinity*temperature on either variable (ANOVA, $p<0.05$; Table 3.3).

In the whole sediment experiments, 21 taxa hatched, although six sediments had no animals emerge under any treatment regime. Three taxa, *Acanthocyclops robustus*, *Nitocra lacustris* and an unidentified juvenile cyclopoid, were found as quiescent copepodids. All remaining taxa were hatched from diapausing eggs. Species richness ranged from 0 to 13 taxa per sediment, with a median value of 2. The rotifers *Synchaeta baltica* and an unidentified *Synchaeta* sp., and copepod nauplii, were the only taxa that hatched exclusively in saltwater. Rotifers and copepods were the most species-rich groups, comprising 76% and 14% of all species hatched from whole sediments, respectively. Copepod nauplii hatched from 6 sediments, and were again considered as a single taxon. One cladoceran, *Daphnia magna*, hatched from ephippial eggs. The experimental salinity treatment had a significant influence on total abundance and species richness hatched (MANOVA, $p < 0.001$; Table 3.4). Univariate

analyses further revealed that increased salinity suppressed both total abundance and species richness (ANOVA, $p < 0.001$; Table 3.4). However, pairwise contrasts revealed that total abundance was significantly greater at 0‰ than for all other treatments (Bonferoni *post hoc* test, $p < 0.001$), while species richness did not differ between the 0 and 8‰ treatments (Bonferoni *post hoc* test, $p>0.05$; see Fig. 3.3). Burial in sediment significantly decreased both total abundance and species richness of hatched taxa, with 0-43% of individuals hatched from isolated resting stages emerging from buried resting stages (MANOVA, $p<0.001$; Table 3.5; Fig. 3.4). Again, this effect was significant for both total abundance and species richness independently (ANOVA, $p<0.001$; Table 3.5).

The two dominant regions for most recent site of ballast uptake were the North Sea (n=14) and west-central Atlantic Ocean (n=8). The Great Lakes basin was the most frequent penultimate source of ballast (n=14). Since total abundance of hatched individuals was significantly related to the density of resting stages (linear regression, $r^2=0.49$, $p<0.001$; Fig. 3.5), relationships to ballast history variables are nearly identical and I only present results for analysis of resting stage density. Resting stage density was weakly related to the salinity of residual ballast water (stepwise multiple regression, $r^2=0.195$, $p=0.013$). All other continuous ballast history variables were found to be unimportant in relation to resting stage density ($p>0.05$). Furthermore analysis of previous regions of ballast uptake determined that only the interaction between the most recent and

penultimate sources of ballast was a significant determinant of resting stage density, after holding residual water salinity as a covariate (ANCOVA, $p<0.05$).

Incorporation of experimental values for resting stage density, viability and sediment tonnage into our propagule pressure model (Eqn. 1) revealed that the distribution of the number of viable resting stages·ship^{-1} is right skewed (mean and median density of 9.8×10^6 and 1.4×10^6, respectively; Fig. 3.6). Resting stages recovered from sediments of 6 ships (17.5%) could not be induced to hatch in the laboratory under any conditions, and were apparently non-viable. Thirty-two percent of ships sampled carried viable resting stages of NIS, at a mean density of 3.0×10^5 estimated from a fitted exponential distribution (95% CI: 1.3×10^5 to 4.9×10^5; Fig. 3.6). The non-linear model generated to predict the number of non-redundant species transported in residual sediments fit the data robustly ($r^2=0.99$). From species-area curves, I predict that up to 150 invertebrate species, including 52 NIS, are transported as resting stages to the Great Lakes each year (Fig. 3.7).

3.5 DISCUSSION

Propagule pressure is emerging as an important factor in the prediction of invasion success in both aquatic and terrestrial environments (e.g., Grevstad 1999a; Lonsdale 1999; Ruiz et al. 2000; Forsyth and Duncan 2001; Drake and Lodge 2004). The propagule pressure hypothesis states that invasion success is directly related to introduction effort. My results indicate that the average NOBOB ship entering the Great Lakes carries about 9.8×10^6 viable, dormant propagules

in residual sediment, one order of magnitude more than the number of live, freshwater propagules estimated to be carried in residual ballast water (MacIsaac et al. 2002b). However, previous studies of ballast water taxa have not described the proportion of propagules that are nonindigenous to the receiving area; I suggest that the actual risk posed by resting stages is much lower than the above numbers indicate, as only ~2.5% of viable resting stages are NIS (i.e., 2.2×10^5). However, considering that approximately 250 NOBOB ships engage in multi-port activities on the Great Lakes each year (Colautti et al. 2003), the expected number of ships entering the system carrying dormant resting stages of NIS is 81 (SD=7.4), or 2.4×10^7 resting stages of NIS·yr^{-1}. Furthermore, this study recorded one extreme event with a single ship carrying 4.5×10^6 resting stages, which was excluded when calculating the population mean in order to allow for application of maximum likelihood estimation. Rare events can be very important in biological invasions (e.g., Lewis 1997), and thus even though we did not use this value in our propagule pressure calculations, its biological significance should not be overlooked. From a management context, identification of the very small number of vessels transporting large egg banks should be a priority.

The Monte Carlo simulation indicates that resting stages of 150 species are likely transported to the Great Lakes each year in NOBOB sediments, of which 52 are NIS. To date, 23 NIS have been recorded from residual ballast sediments. Four of these taxa (17%) are brackish/saltwater species (*Evadne nordmanni*, *Pleopis polyphemoides*, *Synchaeta bacillifera* and *Synchaeta baltica*) that

probably would not survive if introduced to the Great Lakes. One freshwater species, *Bosmina maritima*, is already established in the Great Lakes, possibly owing to transfer in freshwater ballast (De Melo and Hebert 1994). The remaining 18 taxa consist exclusively of rotifers (67%) and cladocerans (33%), and all appear capable of surviving abiotic conditions in the Great Lakes. According to the propagule pressure hypothesis, *Daphnia magna* should have the greatest opportunity to invade the Great Lakes considering that it had the highest frequency and abundance of propagules of NIS. This species has not been recorded in the Great Lakes to date despite having a broad global distribution. Its large body size predisposes it to size-selective predation by planktivorous fishes (Boersma et al. 1999), reducing the likelihood of successful establishment even if introduced to the lakes in large inocula. Interestingly, the next five highest risk NIS (*Brachionus leydigi*, *Filinia passa*, *F. cornuta*, *Asplanchna girodi* and *Cephalodella sterea*), and indeed over half the list of NIS, also possess broad global distributions, yet none have been reported from the Great Lakes. The remaining species (~25%) are almost entirely restricted to the Palaearctic and Oriental biogeographical regions, possibly reflecting current trade patterns of NOBOB ships. Interestingly, no NIS of rotifers have been recorded from the Great Lakes, although Gray et al. (2005) recorded one species from Lake Erie whose status remains uncertain. My hatching studies suggest that rotifers present the predominant invasion risk to the Great Lakes. The lack of reported invasions by this group indicates that resting stages contained in residual sediments are a weak or emerging vector. It is not clear whether copepods also

represent an invasion threat via NOBOB ships, as I was unable to ascertain the species identification of any of the naupliar stages that hatched from diapausing eggs contained in ballast sediments.

In total, I identified 76 distinct taxa from resting stages in residual sediment, with nearly the entire assemblage representing planktonic freshwater species, particularly rotifers (e.g., *Brachionus* spp., *Keratella* spp., *Polyarthra* spp.). This is an indication that reproductive females drawn in with ballast water deposit resting stages directly into ballast sediments, rather than being brought in with disturbed sediments. In addition, rotifers of the genus *Brachionus* were the most common and abundant species in this study. This could be because ballast was taken in areas — such as the lower Rhine River — where *Brachionus* species dominated the planktonic community (van Dijk and van Zanten 1995). However, the predominantly freshwater *Brachionus* species are minor components of other ballast-loading regions, such as the Baltic Sea (Viitasalo et al. 1995; M. Simm and A. Põllumäe, Estonian Marine Institute, Estonia, pers. comm.), and presumably also of coastal areas with high salinity. Thus, the ubiquitous occurrence of these species may be due to an enhanced survivability within ballast tanks associated with broad salinity tolerance (see Bailey et al. 2004; Chapter V). Although 73% of species encountered during this study are considered native to the Great Lakes and do not appear to represent an invasion risk, there is potential for cryptic genetic invasions if novel genotypes are introduced from global ports (e.g., Saltonstall 2002; Turon et al. 2003). Furthermore, the possibility exists that some Nearctic species (e.g., *Daphnia*

retrocurva, Brachionus havanaënsis and Trichocerca multicrinis) could be transferred from the Great Lakes to trading partners overseas.

It is important to note that my measure of propagule pressure is an estimate of the number of dormant individuals transported by NOBOB vessels, rather than the number released into the system. Furthermore, heavily compacted ballast sediments offer little opportunity for direct expulsion of resting stages from ballast tanks. Therefore, the greatest potential for introduction of dormant NIS in residual sediments arises if resting stages are stimulated to hatch inside the ballast tanks of 'multi-port' NOBOB ships, prior to ballast discharge elsewhere on the Great Lakes (Bailey et al. 2003; Chapter II). My measures of viability and species richness may not reflect actual hatch rates inside ballast tanks of operational ships, as all of these experiments were conducted in the laboratory under conditions designed to induce hatching. It is to be expected that a large proportion of resting stages will not hatch within ballast tanks because they do not receive requisite cues for termination of diapause. Although ballast tanks are typically dark, hatching success should not be greatly impacted as previous work has demonstrated that light is not always an essential cue for hatching (Arnott and Yan 2002; Bailey et al. 2003; Chapter II). Instead, the greatest impediment to hatching may come from burial. For example, less than half of all resting stages hatched during my laboratory experiments when immersed in sediment 2 cm thick, as compared to those isolated from sediment (20% average). Thus, the propagule pressure estimated by this study should be considered a maximum value, with the number of propagules available for introduction from resting

stages being at least one order of magnitude lower than that being transported (e.g., ~4.2 x 10^4 resting stages of NIS).

Even if only a small number of resting stages receive hatching cues, it is theoretically possible that these individuals could develop a substantial population within ballast tanks before ballast discharge occurs (see Chapter II; Wonham et al. in review). All rotifers and cladocerans in this study are capable of parthenogenetic reproduction and all have short, multivoltine life cycles, both of which facilitate rapid population growth during warm summer months. Both hatch rate and subsequent population growth rate are affected by temperature (Ruttner-Kolisko 1974; Allan 1976). Thus season could also influence invasion risk posed by vessels, all else being equal. In terms of the 'multi-port' NOBOB ship on the Great Lakes, I expect that invasion risk would be greatest during late summer when temperatures are highest.

Can risk be related to ballast history?

My results suggest that the biological composition of tanks varies through time, as Sorensen's coefficients for repeated ship samples were only marginally higher than that for pairs of tanks from different ships. As a result, ballast history information should be useful for determining differences in community composition. Although resting stage density was significantly related to salinity of residual ballast water it could only explain ~20% of the variability, and thus would not be a particularly informative tool for management decisions; high salinities may simply reflect low occurrence of freshwater taxa in the region of ballast

uptake. There may, however, be some predictive value in the examination of ballast source regions for risk analysis. Although I could not deduce geographic regions of 'high risk' statistically, the significance of the interaction between previous areas of ballast uptake may be an indicator that sediment is retained in ballast tanks from numerous ballast events. In terms of risk of NIS introduction to the Great Lakes, ships loading ballast at ports in the Mediterranean and Black Seas, north-west Pacific, west-central Atlantic Oceans and regions classified as 'other' appear to pose minimal risk because these areas are predominantly saline in nature. By contrast, ships loading ballast in the Baltic or North Seas pose a relatively higher risk owing to the occurrence of freshwater ports in these regions. In this study, seven of the twelve ships carrying NIS had last loaded ballast at ports in the North Sea, while three did so on the Baltic Sea, supporting the hypothesis that these two areas are important donors of NIS to the Great Lakes (Ricciardi and MacIsaac 2000; MacIsaac et al. 2001; Holeck et al. 2004).

While I have described the propagule supply of resting stages associated with NOBOB ships in the transportation stage, measurement error associated with any parameters in the propagule pressure model could have significant consequences and caution must be taken in interpretation of this data. Furthermore, I can make only preliminary estimations of the subsequent success of NIS carried as resting stages at the introduction and establishment stages of the invasion process. On-going, *in situ* studies should help further refine risks associated with release of propagules from NOBOB vessels by assessing hatch

rates of invertebrate resting stages from ballast sediments under operational conditions.

Table 3.1 Percent occurrence and abundance of resting stages collected from 39 ships by taxon, arranged phylogenetically.

Taxon	% occurrence	% abundance
Rotifera	**100**	**77.9**
Asplanchna spp.	66.7	1.0
Brachionus spp.	97.4	76.2
Conochilus spp.	5.1	<1
Filinia spp.	48.2	<1
Synchaeta spp.	5.1	<1
Bryozoa	**61.5**	**<1**
Anomopoda	**76.9**	**9.3**
Bosmina spp.	51.3	<1
Chydoridae	5.1	<1
Daphnia spp.	46.2	7.9
Moina spp.	25.6	<1
Ctenopoda	**2.6**	**<1**
Diaphanosoma spp.	2.6	<1
Copepoda	**76.9**	**2.6**
Indeterminate	**100**	**9.8**

Table 3.2 Species hatched from diapausing eggs in residual ballast sediment that are considered nonindigenous to the Great Lakes. Species are listed in order of decreasing risk, according to propagule pressure and suitability of habitat. Occurrence identifies the number of ships that the species was collected from (out of a possible 35). Abundance lists the cumulative mean number of individuals that emerged from 40 g sediment for all ships on which each species was found. Species hatched in 0‰ medium during laboratory experiments were considered a match for the habitat in the Great Lakes.

Species Name	Occurrence	Abundance	Habitat Match
Daphnia magna[†]	4	6	Y
Filinia passa[†]	4	3.5	Y
Brachionus leydigi[†]	4	3	Y
Filinia cornuta[†]	3	3	Y
Asplanchna girodi[†]	2	1	Y
Cephalodella sterea[†]	1	4.75	Y
Bosmina maritima[§]	1	2	Y
Diaphanosoma orghidani	1	1.25	Y
Brachionus forficula	1	1	Y
Brachionus nilsoni[†]	1	1	Y
Conochilus coenobasis[†]	1	0.5	Y
Diaphanosoma mongolianum	1	0.5	Y
Cephalodella cf. *stenroosi*	1	0.3	Y
Alona rustica	1	0.25	Y
Brachionus bennini[†]	1	0.25	Y
Brachionus diversicornis	1	0.25	Y
Diaphanosoma sarsi	1	0.25	Y
Hexarthra intermedia[†]	1	0.25	Y
Moina affinis[‡]	1	N/A	Y

Table 3.2 (continued)

Synchaeta baltica	1	2.75	N
Synchaeta bacillifera	1	2.25	N
Evadne nordmanni[†]	1	0.5	N
Pleopis polyphemoides[¶]	1	N/A	N

[†]denotes species with broad geographic distribution. [§]denotes species already established in the Great Lakes. Additional references: [‡]Bailey et al. (2003); [¶]S. Bailey, unpublished data.

Table 3.3 Results of two-way MANOVA addressing the effect of experimental temperature and salinity treatment on total abundance and species richness of invertebrates hatched during maximum diversity experiments.

Variable	SS	df	MS	F	*p*
Salinity					
Univariate *F* tests					
Total abundance	26.30	1	26.30	92.50	< 0.001
error	21.32	75	0.28		
Species richness	5.07	1	5.07	125.89	< 0.001
error	3.02	75	0.04		
Multivariate test					
Wilks' lambda = 0.36		2, 74		64.54	< 0.001
Temperature					
Univariate *F* tests					
Total abundance	1.76	1	1.76	6.20	0.015
error	21.32	75	0.28		
Species richness	0.45	1	0.45	11.23	0.001
error	3.02	75	0.04		
Multivariate test					
Wilks' lambda = 0.87		2, 74		5.55	< 0.01
Interaction					
Univariate *F* tests					

Table 3.3 (continued)

Total abundance	0.33	1	0.33	1.18	0.28
error	21.32	75	0.28		
Species richness	0.002	1	0.002	0.06	0.82
error	3.02	75	0.04		
Multivariate test					
Wilks' lambda = 0.98		2, 74		0.89	0.42

SS = sum of squares; MS = mean square.

Table 3.4 Results of MANOVA addressing the effect of experimental salinity treatment on total abundance and species richness of invertebrates hatched during whole sediment experiments.

Variable	SS	df	MS	F	*p*
Univariate *F* tests					
Total abundance	3.20	3	1.07	21.29	< 0.001
error	6.22	124	0.05		
Species richness	2.81	3	0.94	13.00	< 0.001
error	8.94	124	0.07		
Multivariate test					
Wilks' lambda = 0.58		6, 244		12.63	< 0.001

SS = sum of squares; MS = mean square.

Table 3.5 Results of MANOVA addressing the effect of experimental method on total abundance and species richness of invertebrates hatched. Maximum diversity (isolated resting stages) and whole sediment (buried resting stages) experiments are described in detail in methods section.

Variable	SS	df	MS	F	*p*
Univariate *F* tests					
Total abundance	6.22	1	6.22	29.10	< 0.001
error	8.97	42	0.21		
Species richness	15.07	1	15.07	128.40	< 0.001
error	4.93	42	0.12		
Multivariate test					
Wilks' lambda = 0.23		2, 41		69.71	< 0.001

SS = sum of squares; MS = mean square.

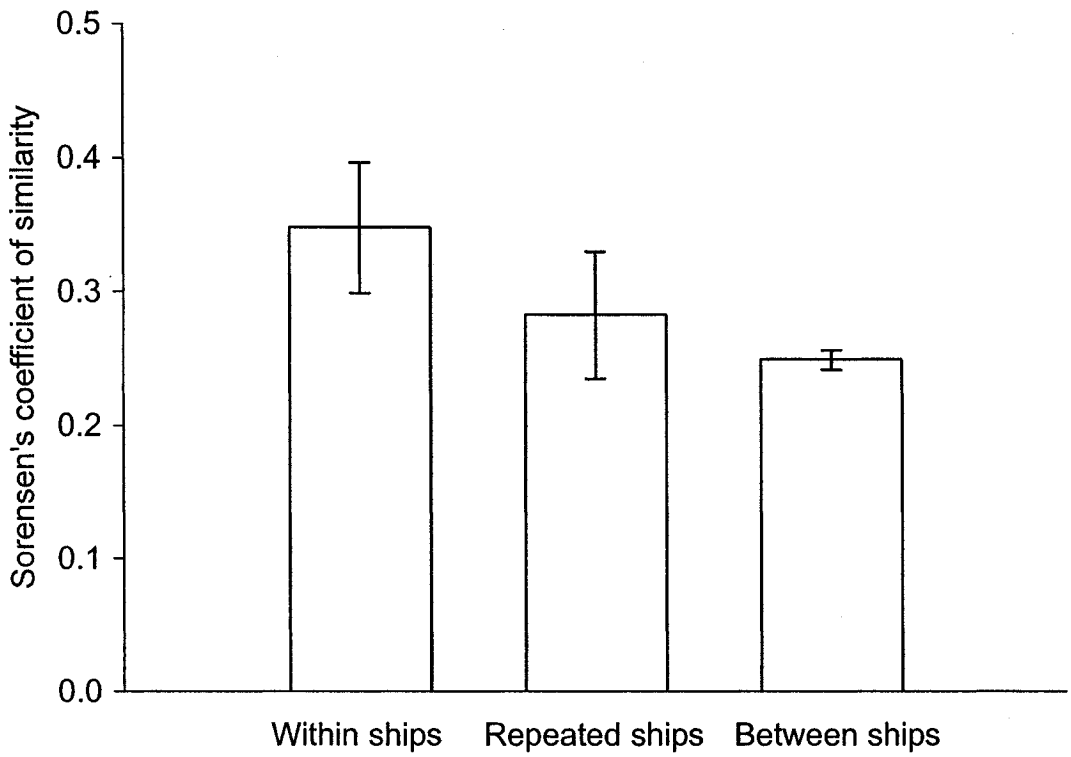

Figure 3.1 Mean (± S.E.) Sorensen's coefficient of similarity for tanks within ships (n=17), tanks on ships sampled repeatedly (n=10), and tanks between ships (n=1000). Sorensen's coefficient typically ranges from zero to one, with higher values indicating greater similarity of samples. Between-ship pairs were selected randomly from possible 47 tanks on 29 ships.

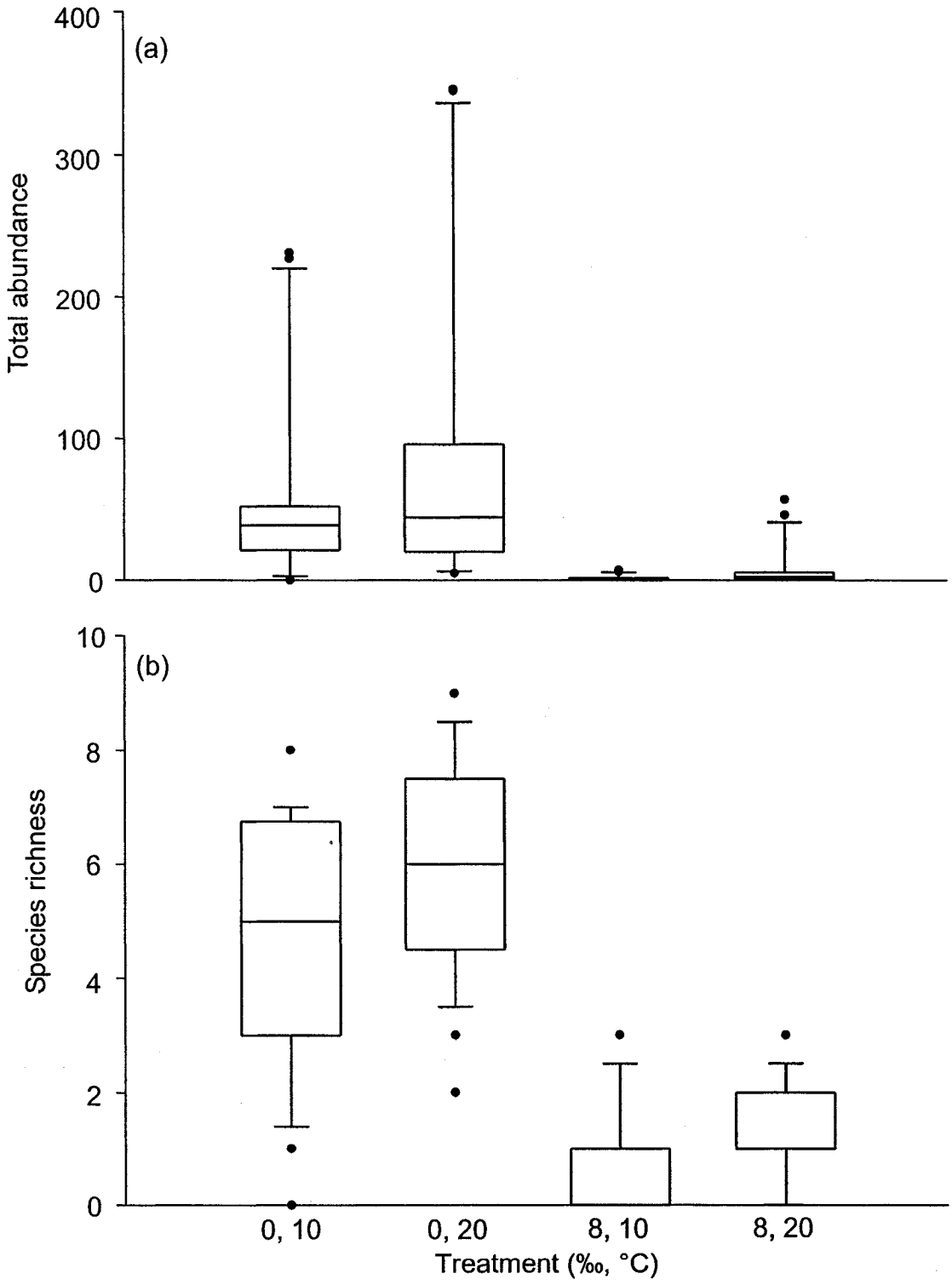

Figure 3.2 Box plots of a) total abundance and b) species richness for organisms hatched from residual sediments of five ballast tanks during replicated maximum diversity experiments.

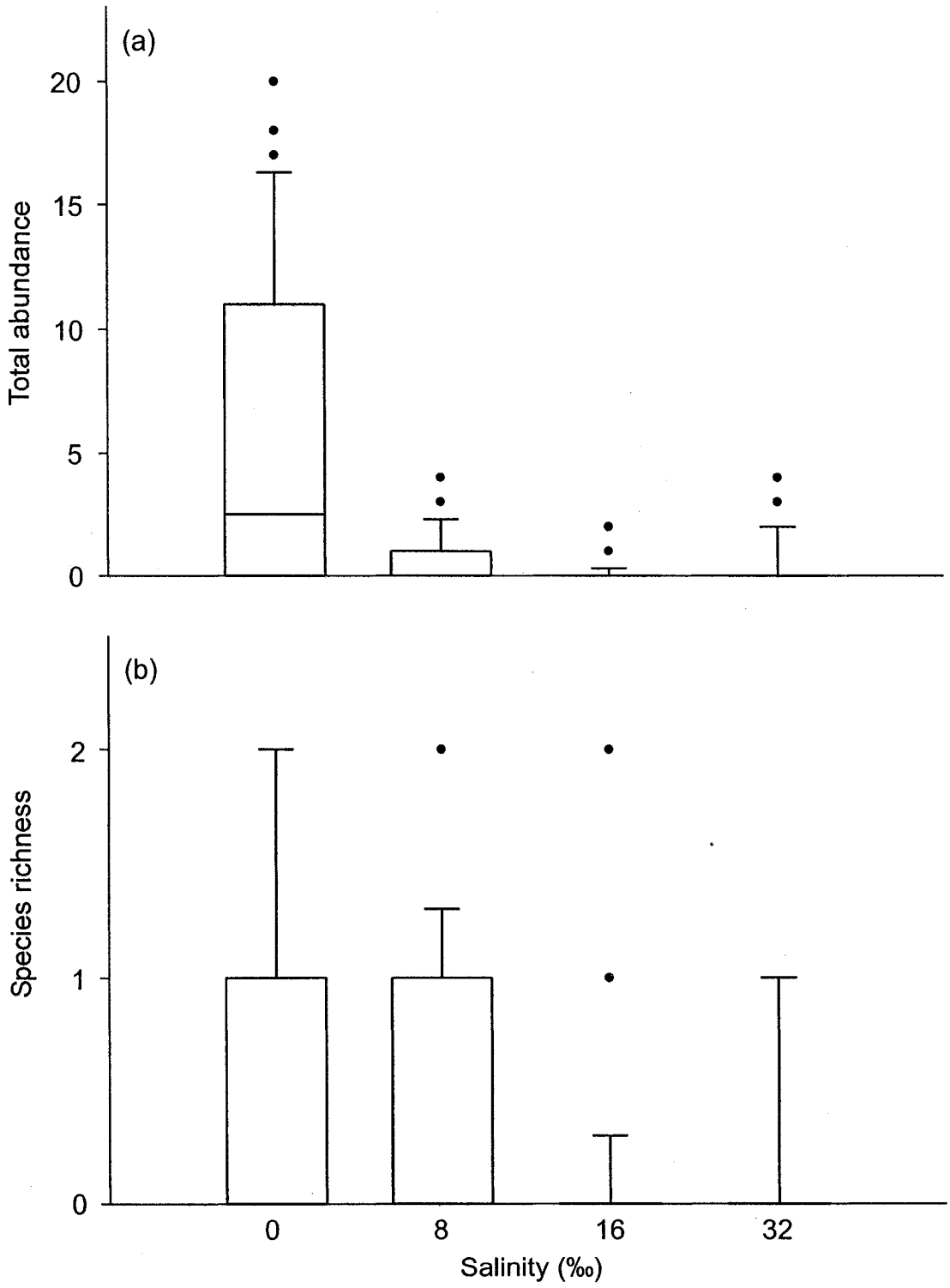

Figure 3.3 Box plots of a) total abundance and b) species richness for organisms hatched from residual sediments of eight ballast tanks during whole sediment experiments. All treatments were incubated at 20°C.

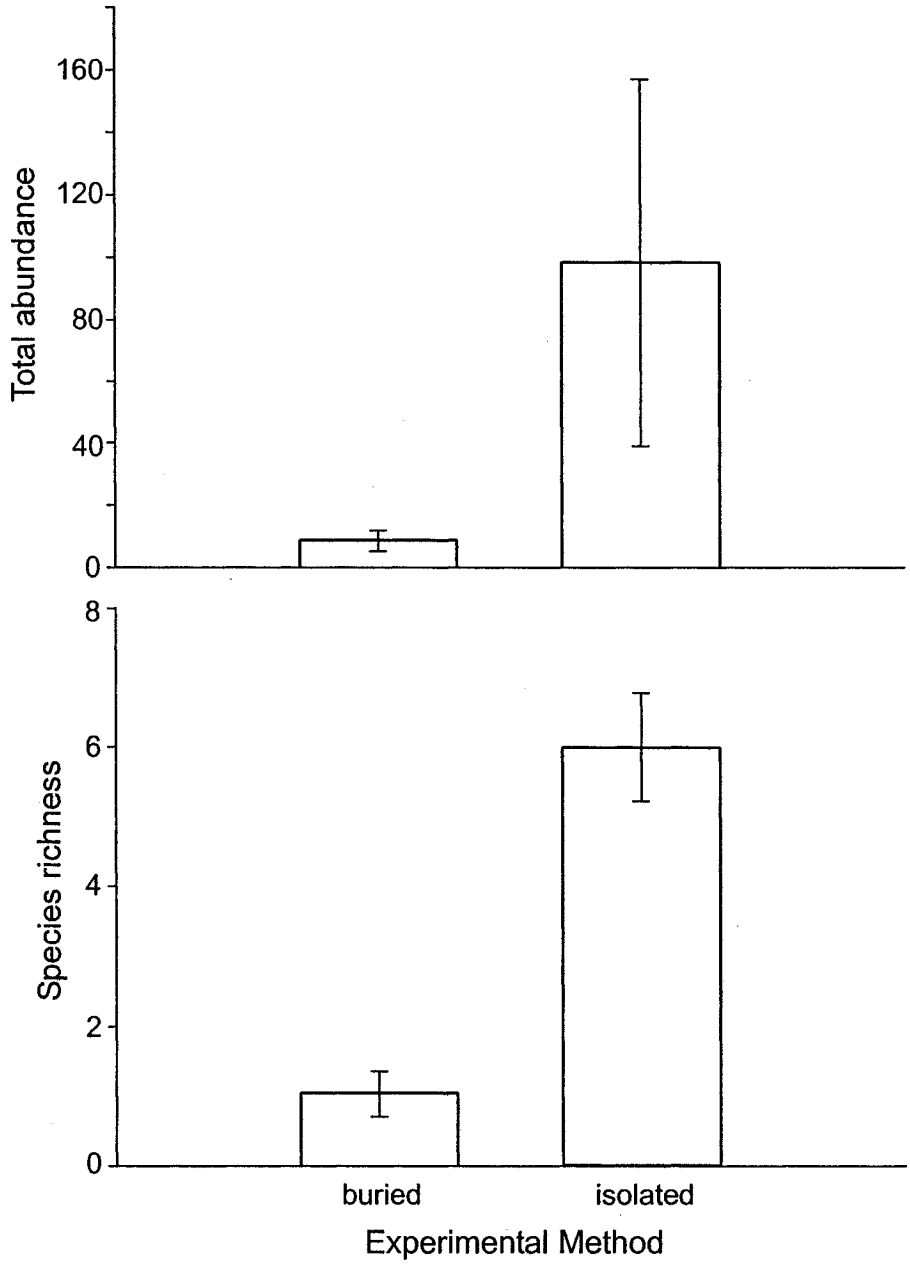

Figure 3.4 Mean (± S.E.) (a) total abundance and (b) species richness of organisms hatched from residual sediments of five ballast tanks in whole sediment (buried) and maximum diversity (isolated) experiments. All replicates were incubated in 0‰ growth media at 20°C. Note scale difference for each y-axis.

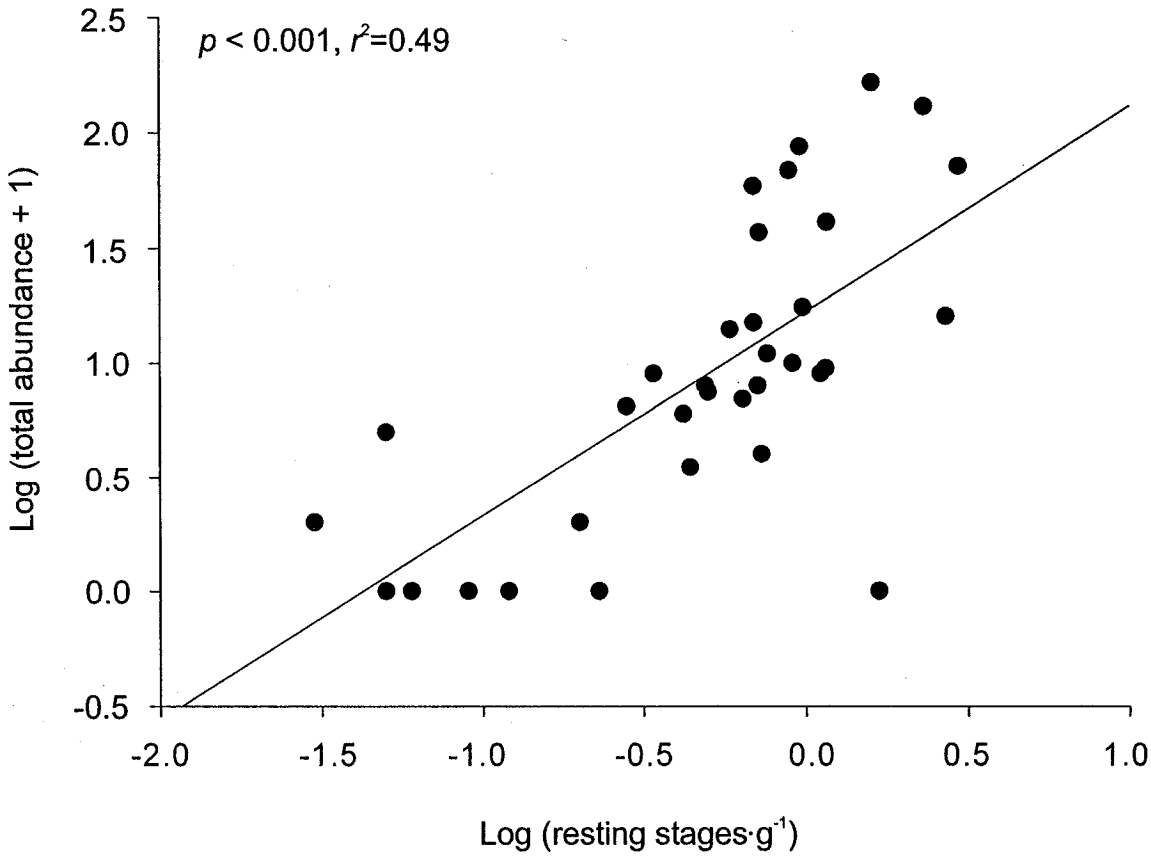

Figure 3.5 Scatterplot of total abundance hatched versus density of resting stages. Both variables were log-transformed prior to analysis. Regression line is y = 0.89x + 1.22.

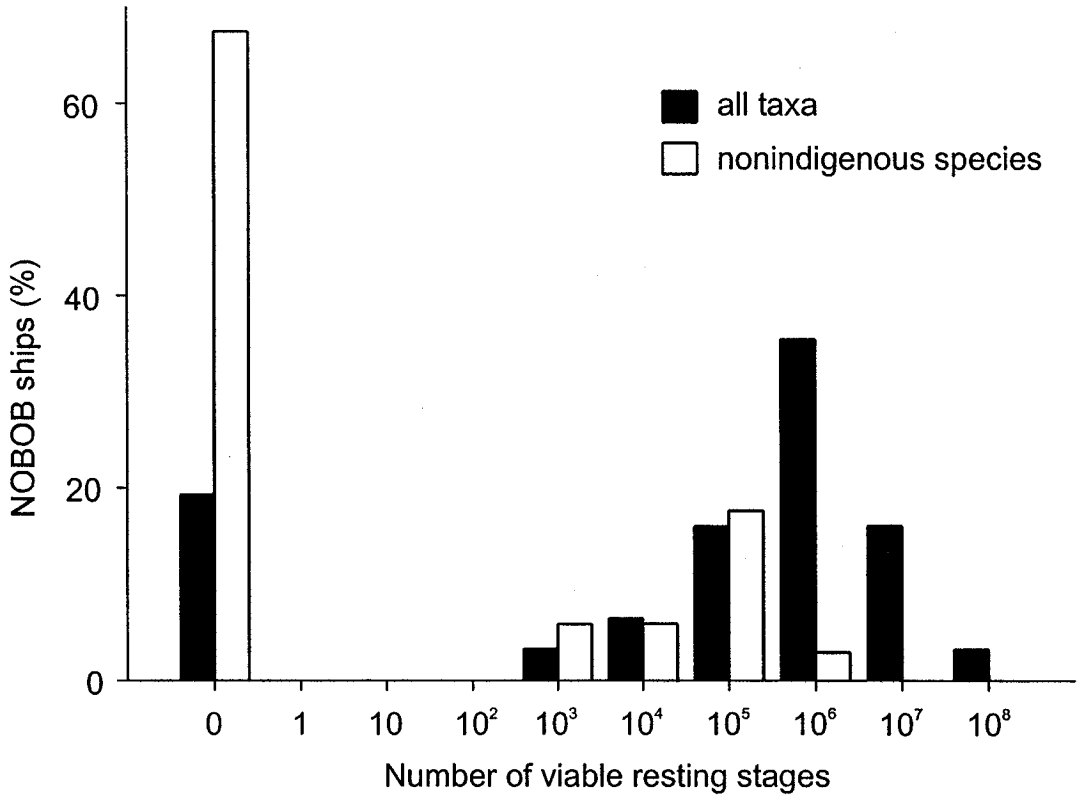

Figure 3.6 Density of viable resting stages transported in sediments of 34 NOBOB ships. Note logarithmic scale on x-axis.

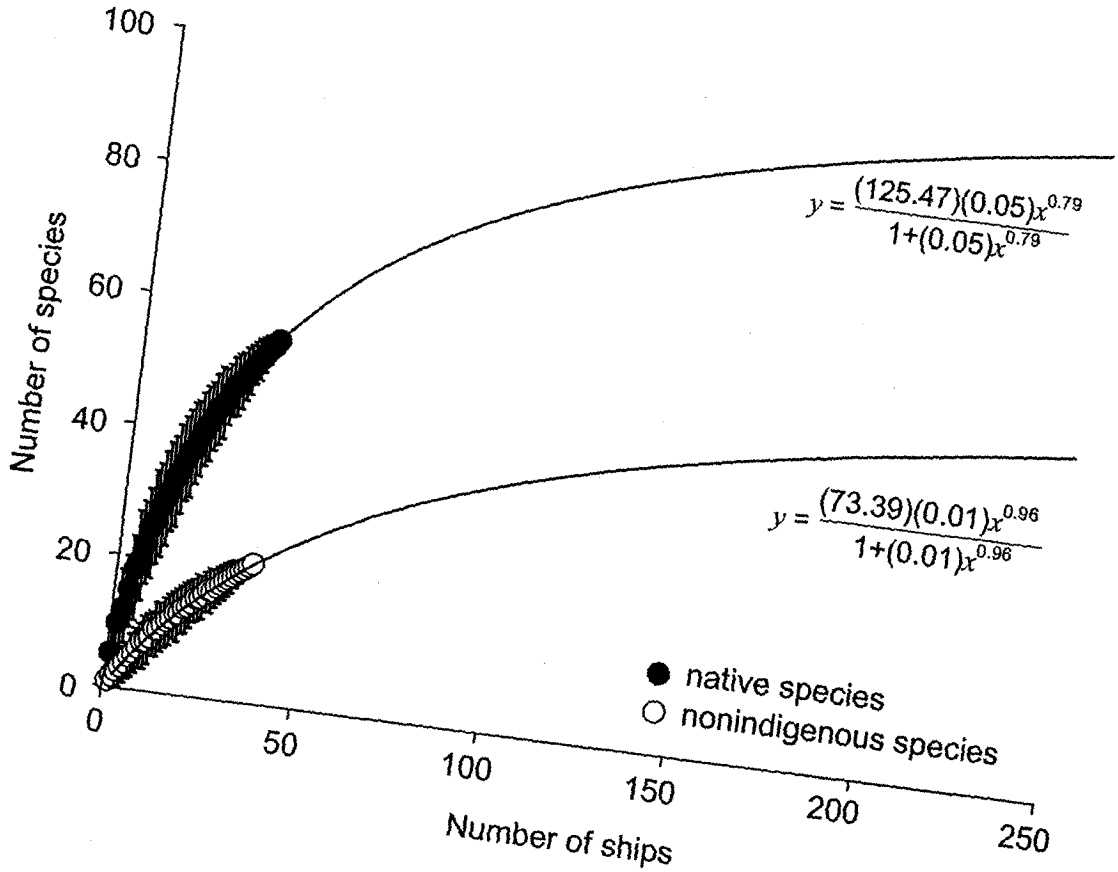

Figure 3.7 Monte Carlo simulations of the cumulative number of non-redundant species (mean ± S.E.) by number of ships sampled. Results were bootstrapped 100 times.

IN SITU HATCHING OF INVERTEBRATE DIAPAUSING EGGS FROM SHIPS' BALLAST SEDIMENT

4.1 ABSTRACT

Ships that enter the Great Lakes laden with cargo carry only residual ballast water and sediment in ballast tanks, are designated 'no ballast on board' (NOBOB), and constitute >90% of inbound traffic. I conducted *in situ* experiments using emergence traps to assess the viability and introduction potential of invertebrate diapausing stages present in ships' ballast sediment. All trials commenced while vessels operated on the lower lakes (Erie, Ontario) and were completed 6 to 11 days later at ports on the upper lakes (Michigan, Superior). In total, eight trials were conducted on four ships using five different ballast sediments. Hatching was observed on every ship, although not from all sediments on all ships. Overall hatch rates were very low (0.5 individuals per 500 g sediment), typically involving activation of <0.05% of total eggs present. Five species of rotifers and copepod nauplii were hatched from ballast sediments, although only one or two species typically hatched from any one sediment. While dormancy is a characteristic enabling enhanced survival during transportation in ballast tanks, it becomes an impediment to introduction. Results from this study indicate, however, that hatching of diapausing eggs contained in ballast sediment of NOBOB ships is a potential mechanism for introduction of new NIS to the Great Lakes. Although inoculum sizes are potentially small (<300 individuals), because reproduction can occur in tanks, and nonindigenous species are

potentially involved in numerous introduction events, the risk posed by this vector is significant.

4.2 INTRODUCTION

Early attempts to understand and predict biological invasions typically consisted of qualitative assessments of species or community characteristics drawn from case studies of successfully established introduced species or invaded ecosystems (Elton 1958; see review by Lodge 1993a). General patterns elucidated during this research provided a framework for development of invasion theory, although few of the studies were quantitative or experimental and most failed to consider alternative explanations for observed patterns (Lodge 1993a; Kolar and Lodge 2001; Colautti and MacIsaac 2004). Recent studies designed to identify life history differences between successful and unsuccessful invasions of nonindigenous species (NIS) have focused on the stage of the invasion (e.g., Wonham et al. 2000; Kolar and Lodge 2001, 2002). These studies recognize that successful invasions encompass a series of different stages including transport, introduction, establishment and, in some cases, spread. Because uptake and transportation by a vector are the earliest stages in the sequence, efforts have been made to link introduction effort or 'propagule pressure' to invasion success (Veltman et al. 1996; Duncan et al. 2003; Rouget and Richardson 2003; see also Colautti et al. 2005a). Introduction effort itself has two components: the inoculant size for a given introduction event and the frequency of inoculation events (see Kolar and Lodge 2001; Colautti and

MacIsaac 2004). Although not widely tested in aquatic ecology, available evidence generally supports the propagule pressure hypothesis (Colautti et al. 2005a).

Ship-mediated vectors – hull fouling, ballast water and sediments – are regarded as principal pathways for unintentional introductions of aquatic organisms worldwide (e.g., Carlton 1985; Ruiz et al. 2000; Leppäkoski et al. 2002; Grigorovich et al. 2002, 2003; Hayes and Sliwa 2003). Organisms may be introduced by ship-mediated vectors if they can survive the physical, chemical and biological rigours associated with uptake and discharge of ballast water, in addition to long-distance transport (Carlton 1985). Studies of ballast water invasions by nonindigenous invertebrates to the Great Lakes have suggested that the ability of species to form dormant or diapausing life stages should enhance survivability through the transportation stage (stage I, *sensu* Colautti and MacIsaac 2004; Holeck et al. 2004). Analysis of the invasion history of the Great Lakes since 1959 confirmed that 15 of 19 NIS of crustaceans that successfully invaded are able to produce resting or diapausing stages (Bailey et al. in review). However, the dense nature of residual ballast sediments, combined with their propensity for accumulation in tanks, suggests that diapausing stages within sediments will not ordinarily be expelled from tanks during normal operation. Thus, the ability of diapausing stages to transition directly from transportation (stage I) to introduction (stage II) is probably limited. A much more likely mechanism for introduction of NIS from residual sediment consists of hatching of viable diapausing eggs while the ship is in ballast, followed by

discharge of planktonic taxa during deballasting operations. Standard operations of 'no ballast on board' (NOBOB) vessels may induce hatching of diapausing eggs when ships visit multiple ports on the Great Lakes (Bailey et al. 2003; Chapter II). These vessels enter the system loaded with cargo and carry only residual water and sediment in ballast tanks. NOBOB vessels load Great Lakes' water as ballast to maintain vessel trim and stability after offloading cargo, usually at ports on the lower lakes (Colautti et al. 2003). In Chapter II, I proposed that the uptake of oxygenated freshwater stimulates diapausing eggs in ballast sediments to hatch, facilitating release of planktonic taxa when the mixed ballast water is discharged at the last port-of-call.

All experiments conducted to date on viability of invertebrate resting stages have been performed in the laboratory under controlled conditions (Bailey et al. 2003, 2004; Gray et al. 2005). Here I seek to estimate the number of propagules passing through the introduction phase (stage II) with *in situ* studies of hatching by diapausing eggs contained within residual sediments of transoceanic ships operating on the Great Lakes. Specifically, I deploy a series of emergence traps containing defined ballast sediment within ballast tanks of operational vessels to determine if diapausing eggs are stimulated to hatch with the addition of Great Lakes water to ballast tanks.

4.3 METHODS

Use of emergence traps in ballast tanks allows incubation of diapausing eggs under conditions closely approximating those of the ballast tanks. Through

careful deployment of control traps, it is possible to differentiate individuals hatched from ballast sediments from contaminants in the tanks, and to determine whether conditions were suitable for aerobic species. Experiments using emergence traps were conducted on four ships in October 2002, and July and September 2003 (see Table 4.1). Two trials were conducted inside the same tank on each ship, each using a different ballast sediment. In total, five sediments were used for the eight trials. Candidate sediments were collected in December 2001, June 2002 and June and July 2003 from individual ballast tanks of NOBOB ships operating on the Great Lakes (methodology as in Chapter II); sediments used here were selected on the basis of high egg density to maximize opportunities for hatching of diapausing eggs (see Chapter III for distribution of egg densities found in ballast sediments). Subsamples of each sediment were removed to characterize the density and diversity of diapausing eggs in the laboratory before usage of sediments in emergence trap experiments (see Chapter III for laboratory methods). Sediments were stored in the dark at 4°C until the onset of *in situ* trap experiments.

Trap design and deployment — Simple, low-cost emergence traps were constructed from standard PVC plumbing components to monitor *in situ* hatching of diapause eggs from residual ballast sediments. Emergence traps were designed to allow ballast water to flow through each experimental chamber, while excluding organisms present in the surrounding water and retaining any organisms hatched inside traps. Each emergence trap was built from a 15 cm (interior diameter) pipe cap with a threaded lid, having a surface area of

approximately 180 cm^2. Bolts were put through the bottom of the pipe cap to secure the trap to a rectangular PVC platform and sealed with silicone glue (Fig. 4.1). Twelve holes of approximately 2.5-4 cm diameter were drilled through the lid and wall of each trap, and were subsequently covered with Nitex plankton mesh (mesh sizes given below). Mesh was attached to the trap housing using clear PVC cement and edges were sealed with silicone glue. After construction, traps were left to cure for 48h and were subsequently rinsed with deionised water to remove any coarse debris and soluble compounds left by the glues.

Trap platforms, each carrying three linearly spaced emergence traps, were secured to the bottom surface of the ballast tank using cable ties threaded through drainage holes in the ballast tank's interior structure. After all platforms were moored, each trap was loaded with 500 g residual ballast sediment (approximately 2 cm in depth) and trap lids secured. After our crew exited the tanks, ballast water was loaded into the tanks such that all traps were submerged. Ships thereafter resumed normal operation, and offloaded cargo at one to three additional ports before discharging all ballast water and loading outbound cargo at the final port-of-call. Zooplankton composition of ballast water during transit was monitored by collecting three replicate net hauls of ~100-400 L volume, using a 0.5 m-diameter 30 μm plankton net, preserved in 95% ethanol. Net samples were taken inside ballast tanks immediately after traps were submerged and at two or three subsequent ports, including the final port before ballast was discharged. Each net sample was later scanned under a dissecting microscope and representative taxa were identified with a compound microscope

at up to 1000x magnification. In addition, temperature readings were measured near tank bottom at all ports where net samples were drawn using a Hydrolab DataSonde 4a.

On the initial ship, traps were constructed using 34 μm Nitex mesh. Six traps were used for each of two sediment types. One trial consisted of five experimental replicates (precharacterized sediment), and a negative control trap (autoclaved sediment) to monitor for introduction of species from the ship's ballast water. The second trial had four experimental replicates, and negative and live control traps. The latter trap contained the same non-autoclaved sediment as was used for experimental replicates, to which 40 oligochaetes, *Lumbriculus variegatus*, and 40 amphipods, *Hyalella azteca*, were added. These species served as sentinels of sediment toxicity and anoxia in the traps (e.g., Sprague 1963; Putzer et al. 1990; Dermott and Munawar 1992; Nebeker et al. 1992; Phipps et al. 1993).

The experimental design was modified slightly for all subsequent trap experiments in an effort to improve water flow through traps, and to increase statistical power. These experiments used traps with 53 μm mesh, and six experimental replicates per trial. Each trial had its own negative and live controls, the latter containing 20 *L. variegatus* and 20 *H. azteca.* In addition, a positive control was included for each trial, consisting of diapausing eggs isolated from 500 g sediment using a sugar flotation protocol (Bailey et al. 2003; Chapter II); this control served to assess whether hatching and survival of taxa were similar with and without sediment present. Prior laboratory experiments illustrated that

diapausing eggs had higher hatch rates when separated from sediments (Chapter III). A lack of hatching in this control trap would highlight possible toxicity effects associated with the sediment, or other inhospitable conditions inside the trap; conversely, presence of hatching in positive controls, when hatching is absent in experimental replicates, would indicate effects associated with burial of eggs in sediment.

Trap recovery and analysis — Traps were recovered at the terminal port-of-call after ballast water had been discharged. Approximately 2.5 cm of water, which remained inside traps below the drainage holes, was collected by large-mouth pipette and filtered through 30 μm mesh. The filtrate was preserved using 95% ethanol for later enumeration and identification of invertebrate taxa. Sediment was subsequently recovered from each trap and preserved in 95% ethanol; taxa associated with the sediment were isolated for enumeration and identification using the colloidal silica Ludox® HS40 (Burgess 2001). Negative and positive controls were recovered and analysed in the same manner, except that positive controls consisted solely of a water sample and thus the Ludox method was not required. After recovery, all emergence traps were inspected for integrity; four experimental replicates were excluded from analysis due to visible tears in plankton mesh. Live control traps were surveyed to determine the number of oligochaetes and amphipods that remained alive.

For enumeration of hatched taxa, any taxa recovered from emergence traps that do not possess diapausing stages (e.g., bdelloid rotifers, bivalves) or mature forms that could not have developed within the transit timeframe (e.g.,

copepodids) were excluded from analyses since these organisms were likely introduced with the sediment at the onset of the experiment. In addition, analysis of negative controls indicated that some organisms present in the Great Lakes ballast water had infiltrated the plankton mesh on emergence traps. As a result, all taxa present in either negative control for each ship were subtracted from results of experimental replicates. Furthermore, to be conservative, any recovered taxa not identified during laboratory experiments using the same sediments (under ideal growth conditions) were excluded from analysis. My analyses are therefore based on conservative estimates of *in situ* hatch rates and likely underestimate actual richness and abundance of hatched taxa. Total abundance of organisms hatched from *in situ* experimental replicates was compared to that of laboratory characterization experiments using a Mann-Whitney U-test (Systat 8.0, SPSS, Inc., 1998). Since laboratory experiments were conducted using only 40 g (as opposed to 500 g) sediment replicates, total abundances of hatched species were extrapolated to 500 g before analysis. I also compared species richness of laboratory and *in situ* hatch rates using a Mann-Whitney U-test.

Estimation of inoculum size — To determine the risk of introduction posed by invertebrates hatched from diapausing eggs carried by transoceanic vessels, I calculated the average rate of hatching per 500 g replicate for the eight *in situ* trials. This rate was used to estimate the number of individuals hatching across an entire ship, given that a single ship typically carries 10-15 tonnes of residual sediment (P.T. Jenkins, unpubl.; Chapter III). As hatching rate may be affected

by surface area, I also produced estimations based on surface area of traps, scaled by surface area of residual sediment across a ship, using the assumption that residual sediment is uniformly distributed across the ship hull at a depth of 2.5 cm. Finally, I used occurrence and abundance of NIS recorded from previous ballast sediment characterization studies to estimate the typical inoculum size of taxa that would pose an invasion threat to the Great Lakes.

4.4 RESULTS

Emergence traps remained submerged for 6 to 11 days, depending on ship schedule. Water temperature near the bottom of ballast tanks ranged widely, primarily due to season and storm events, with average temperatures during voyages ranging between 16.5 and 20.6°C (Table 4.1). A fraction of live control animals were not recovered at the end of the first three experiments due to procedural errors during processing; however, 100% of live control animals were recovered after the fourth and final *in situ* experiment. All live control animals that were recovered were alive, indicating that environmental conditions within traps were sufficient to support life for the duration of each voyage. In total, 19 individuals were hatched from 41 experimental replicates, producing an average hatching abundance of 0.5 (SD=1.0) individuals per 500 g replicate (Table 4.2). Hatching occurred in six of eight trials. Both trials without hatching occurred on separate ships; thus hatching occurred in at least one experimental replicate on every ship. Species that hatched included the rotifers *Brachionus calyciflorus*, *Cephalodella catellina*, *Keratella tecta*, *Synchaeta oblonga* and *Trichocerca*

pusilla, and copepod nauplii (Table 4.2). None of the individuals recovered from experimental replicates were observed in a reproductive condition.

Diapausing eggs were not as likely to hatch *in situ* as under laboratory conditions. Both total abundance and species richness of organisms hatched were significantly lower *in situ* than in laboratory characterization trials (Mann-Whitney *U*-test, $p<0.001$; Fig. 4.2). In addition, the effect of burial had a significant impact on the number of eggs that hatched (Mann-Whitney *U*-test, $p<0.001$; Fig. 4.3), although this could only be tested for the laboratory experiments since positive controls on ships were not replicated.

Hatching also occurred in all six positive controls, supporting the hypothesis that abiotic conditions in ballast tanks were favourable and that hatching was inhibited in experimental replicates by some factor associated with the sediment. In total, 1561 individuals were recovered from positive controls (mean 260 ± 425), although this number likely included parthenogenetic offspring (see Table 4.2). Species hatched in positive controls include the rotifers *Brachionus angularis*, *B. budapestinensis*, *B. calyciflorus*, *B. diversicornis*, *Keratella tecta* and *Trichocerca stylata,* and copepod nauplii (Table 4.2).

Estimation of inoculum size — The rate of hatching recorded during this study ranged from 0.01-0.04%. Extrapolations based on sediment volume and surface area suggest the sediments used in these experiments could release approximately 7000 to 15000 individuals per ship under similar environmental conditions (typically June - October). However, previous work suggests that only ~2.5% of resting stages transported in residual sediments are from species

nonindigenous to the Great Lakes (Chapter III). Consequently, the inoculum size of NIS introduced via this vector is probably much lower than the total number estimated by this study, at 175-375 nonindigenous individuals per ship. Furthermore, since ships with nonindigenous taxa present typically carry one or two NIS each (S.A. Bailey, unpubl.), this results in an inoculum size of only 87-375 individuals per taxon.

4.5 DISCUSSION

Previous studies estimate that only a small proportion (~10%) of invaders will survive passage from the transportation stage to the introduction stage, with most organisms dying in transit (Carlton 1985; Williamson and Fitter 1996; Kolar and Lodge 2001). However, diapausing eggs likely enhance survivability of invertebrates during transportation, with up to 92% of eggs collected from residual sediments being viable in laboratory studies (Bailey et al. 2003; Chapter II). But does this mechanism that increases survival during the transportation stage also facilitate successful introduction? Through the use of *in situ* emergence trap experiments, I was able to demonstrate that diapausing eggs can hatch from sediments inside ballast tanks of operational ships, albeit at very low rates.

Although all of the species hatched in these *in situ* trials are cosmopolitan and appear to pose little or no invasion risk, they could pose a risk for genetic invasion, as many plankton species differ genetically between continents. However, as shipping activities have been translocating thousands of species, for

hundreds of years, the broad geographical distributions of many taxa today may actually be the result of earlier introductions (Gollasch et al. 2002; Carlton 1985, 2003). Nonetheless, the NIS *Brachionus diversicornis* was hatched from positive controls on two voyages, confirming that NIS with restricted distributions are present as diapausing eggs at extremely low abundance (see Chapter III). The fact that *B. diversicornis* was not hatched from experimental replicates may be a result of the small volumes of sediment used. Conversely, burial in sediment may have precluded hatching cues from inducing hatching in this species.

Only 0.5 diapausing eggs hatched per 500 g replicate during these experiments. This value is less than 0.05% of the total number of eggs present in the experimental sediments. Despite the fact that NOBOB vessels typically carry 10-15 tonnes of sediment, the probability that NIS will be present and receive hatching cues is small, giving an estimated inoculum size of 87-375 individuals per taxa. Furthermore, as the sediments used in this study were selected for high egg density, this is likely a greater inoculum than that presented by most ships entering the system.

Propagule pressure is based not only on the inoculum size, but also the frequency of inoculations. Approximately 250 NOBOB vessels conduct 'multi-port' operations on the Great Lakes each year, providing suitable conditions for hatching and introduction of resting stages (Colautti et al. 2003). As roughly 32% of these vessels will carry resting stages of NIS (Chapter III), this translates to approximately 5.7×10^3 to 3.0×10^4 nonindigenous individuals introduced via the residual sediment vector per year, assuming that all hatched individuals will be

discharged from ballast tanks. Considering that a single NOBOB vessel carries ~10^5 individuals of NIS via residual ballast water (Duggan et al. in review), the relative importance of diapausing stages in sediments appears much lower.

Since the prolonged existence of temporal zooplankton is dependent on the formation of a sexually-produced diapausing egg bank, Allee effects (i.e., zero or negative growth of small populations owing to density-dependent population dynamics) may impact establishment success of nonindigenous zooplankton introduced to the Great Lakes. However, in a modelling analysis, Drake (2004) estimated that inoculum sizes as small as ten parthenogenetic individuals may result in successful establishment if given enough time to produce a large population before the onset of sexual reproduction. Activities of NOBOB vessels also seem to fit the optimum release strategies calculated for terrestrial biological control insects by participating in numerous, small-sized release events (as discussed in Grevstad 1999b). Furthermore, since ballast-mediated introduction events are spread out over both time and space, risks associated with environmental stochasticity will be reduced, indicating that the inoculum sizes estimated by this study pose a risk of invasion.

During the course of this study, I recorded one individual of the NIS *Brachionus leydigi* from the Great Lakes' ballast water loaded on voyage two. This species has been observed as a rare component of diapausing egg fauna in residual ballast sediments of previous studies (Chapter III). However, since residual sediments generally do not accumulate in upper wing tanks, this individual probably did not hatch from sediments within the tank but may be the

result of a previous introduction to Hamilton Harbour from ballast discharge by a transoceanic vessel. As only one individual was recovered from plankton samples, I cannot determine whether the species has established in Hamilton Harbour, but this finding indicates that introductions of NIS of rotifers may already have occurred.

I have not adjusted inoculum size for increases due to reproduction since there was no evidence of reproduction by the individuals hatched in experimental replicates during this study. Reproduction rates of parthenogenetic taxa are affected by numerous factors, such as temperature, food quantity and quality, and genetic composition (Wallace and Snell 2001). Assuming exponential growth (Taylor 1988) and published life history parameters for *Brachionus calyciflorus* at 16-20°C (Pourriot and Rougier 1997; Wallace and Snell 2001), I determined that 15-45 individuals may have been the founding (hatching) population size for the 1073 individuals recovered from the positive control during voyage four. The occurrence of reproduction in positive control replicates during voyages three and four suggest that ballast tanks can provide suitable conditions for parthenogenetic reproduction, at least during warmer months. Time may have been a constraining factor for reproduction in experimental replicates as eggs isolated from sediments probably hatched a number of days earlier than those buried in sediments. Alternatively, toxicity or hypoxia caused by high biological oxygen demand of ballast sediments may have prevented reproduction in experimental replicates as the plankton mesh may have prevented adequate water flow and oxygen renewal. If this is the case, this study underestimates

inoculum sizes since hatched organisms inside ballast tanks may initiate reproduction under more favourable abiotic conditions.

This study provides empirical support for the hypothesis that different life history characteristics are beneficial during various stages of the invasion process. While dormancy is a characteristic enabling enhanced survival during transportation, it becomes an impediment at the introduction stage, as less than 0.05% of individuals will likely pass from the transportation stage to the introduction stage under conditions experienced in ships' ballast tanks. In contrast, live planktonic animals probably have low survivability in ballast tanks but high opportunity for introduction with deballasting of water (see MacIsaac et al. 2002b). As environmental and demographic stochasticity will further reduce the number of individuals successfully transitioning from the introduction stage (stage II) to the establishment stage (stage III) of the invasion process, the risk of invasion via diapausing eggs in residual ballast sediments appears to be low. However, the assumption that sediments — and the resting stages contained therein — are not being deposited directly into the Great Lakes, either during regular deballasting or tank cleaning operations, must be validated to ensure that this risk is not underestimated. Dry-dock cleaning of sediments from ballast tanks should be carefully managed, since it can provide a direct route for discharge of diapausing eggs into adjacent waterbodies.

Table 4.1 Summary of transit dates and locations for *in situ* ship experiments. Two trials were run inside the same tank of each ship. Average temperature ± SD, as measured near tank bottom, was calculated from 3-4 points of time across voyage. Tank types: FP – forepeak, UW – upper wing tank, DB – double-bottom. Number of traps column lists number of experimental replicates, negative, positive and live control traps for each sediment type; traps excluded from analysis also excluded here.

Voyage	Start location	End location	Tank Type	Average temp (°C)	Sediment	No. traps (E, N, P, L)
1	Windsor, ON	Milwaukee, WI	FP	16.5 ± 1.6	A	5, 1, 0, 0
	Oct 5, 2002	Oct 11, 2002			B	4, 1, 0, 1
2	Hamilton, ON	Thunder Bay, ON	UW	20.6 ± 2.6	A	4, 1, 1, 1
	July 2, 2003	July 13, 2003			C	5, 1, 1, 1
3	Hamilton, ON	Milwaukee, WI	UW	20.4 ± 2.1	B	6, 1, 1, 1
	July 14, 2003	July 24, 2003			C	6, 1, 1, 1
4	Cleveland, OH	Duluth, MN	DB	18.4 ± 5.4	D	6, 1, 1, 1
	Sept 15, 2003	Sept 26, 2003			E	5, 1, 1, 1

Table 4.2 Summary of hatching results for *in situ* emergence trap experiments. Egg density is average number ± SD of diapausing eggs per 500 g sediment placed in traps at start of voyage. Hatching success gives mean ± SD number of individuals hatched per successful experimental replicate (n in parentheses). Positive control lists number of individuals hatched from single replicate with isolated eggs; * indicates presence of adult females carrying parthenogenetic eggs.

Voyage	Species Hatched	Egg Density	Hatching Success	Positive Control
1	**sediment A**	1114 ± 103		
	Brachionus calyciflorus		1 (1)	n/a
	copepod nauplii		1 ± 0 (2)	n/a
	sediment B	1840 ± 385		
	no hatching		0	n/a
2	**sediment A**	1114 ± 103		
	B. angularis		0	1
	B. budapestinensis		0	1
	B. calyciflorus		1 (1)	9
	sediment C	538 ± 81		
	B. budapestinensis		0	1
	B. calyciflorus		0	13
	B. diversicornis		0	1
3	**sediment B**	1840 ± 385		
	B. angularis		0	38*
	B. calyciflorus		0	51*
	Cephalodella catellina		1 (1)	0
	Synchaeta oblonga		1 ± 0.6 (3)	0
	Trichocerca pusilla		1 ± 0 (2)	0

Table 4.2 (continued)

	sediment C	538 ± 81		
	copepod nauplii		1 (1)	9
	Trichocerca stylata		0	2
4	**sediment D**	2966 ± 276		
	B. angularis		0	10
	B. budapestinensis		0	1
	B. diversicornis		0	1
	B. calyciflorus		5 (1)	1073*
	sediment E	1605 ± 80		
	B. angularis		0	59*
	B. calyciflorus		0	289*
	Keratella tecta		2 (1)	2
Cumulative Total			**19 (13)**	**1561**

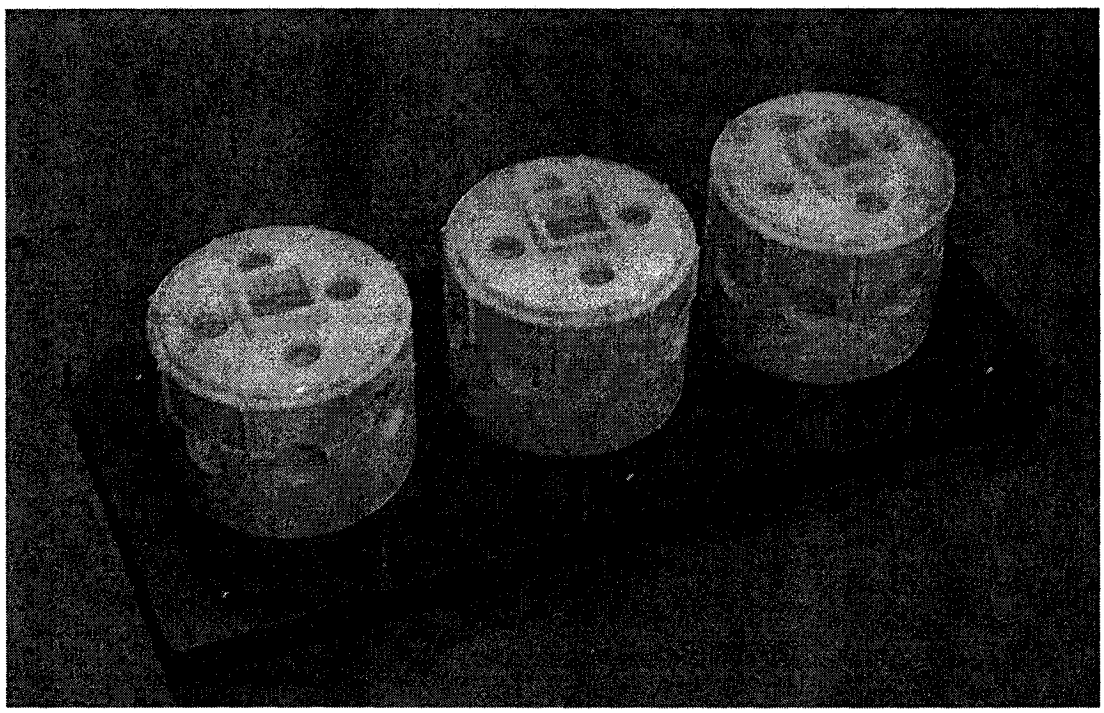

Figure 4.1 Photograph of three emergence traps bolted to PVC platform. Holes in lids and sides of traps are covered with 53 μm Nitex plankton mesh. Holes at platform corners were used to secure traps to ballast tank's internal structure.

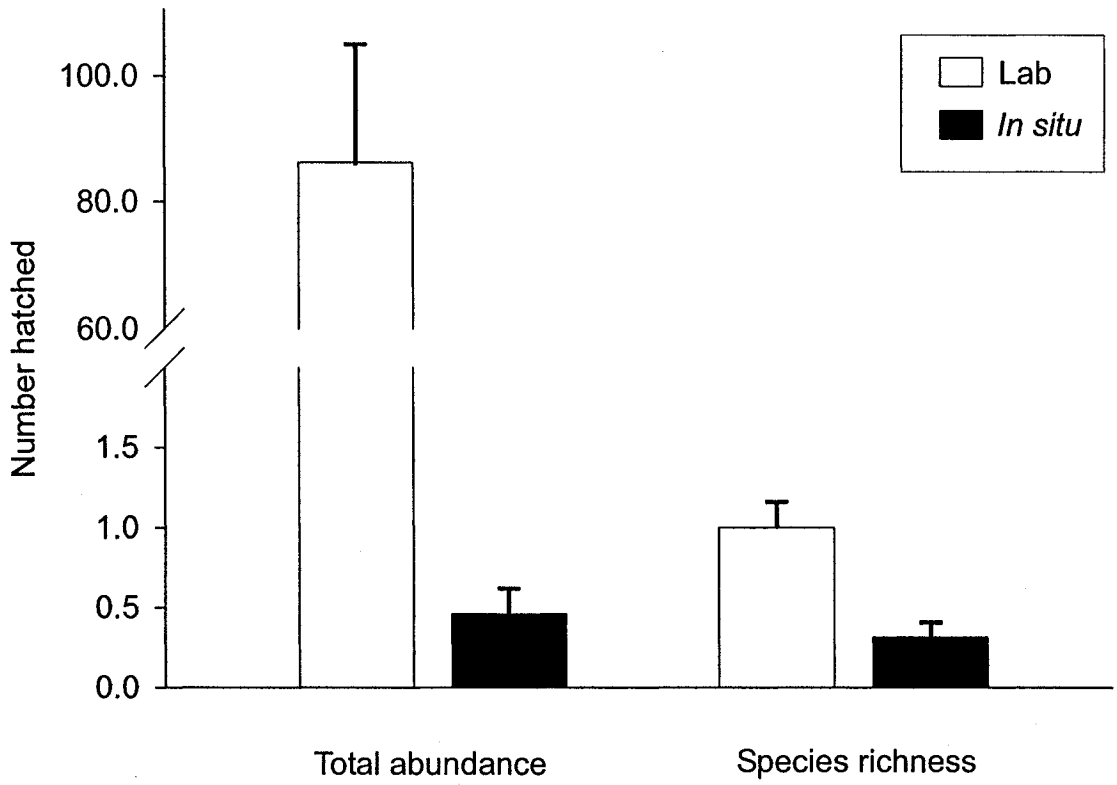

Figure 4.2 Total abundance and species richness of taxa hatched from residual ballast sediments in laboratory and *in situ* studies. Total abundance is number of individuals hatched per 500 g sediment, while species richness is number of taxa hatched per 40 g sediment in lab, and 500 g sediment in ship. Note scale break in y-axis.

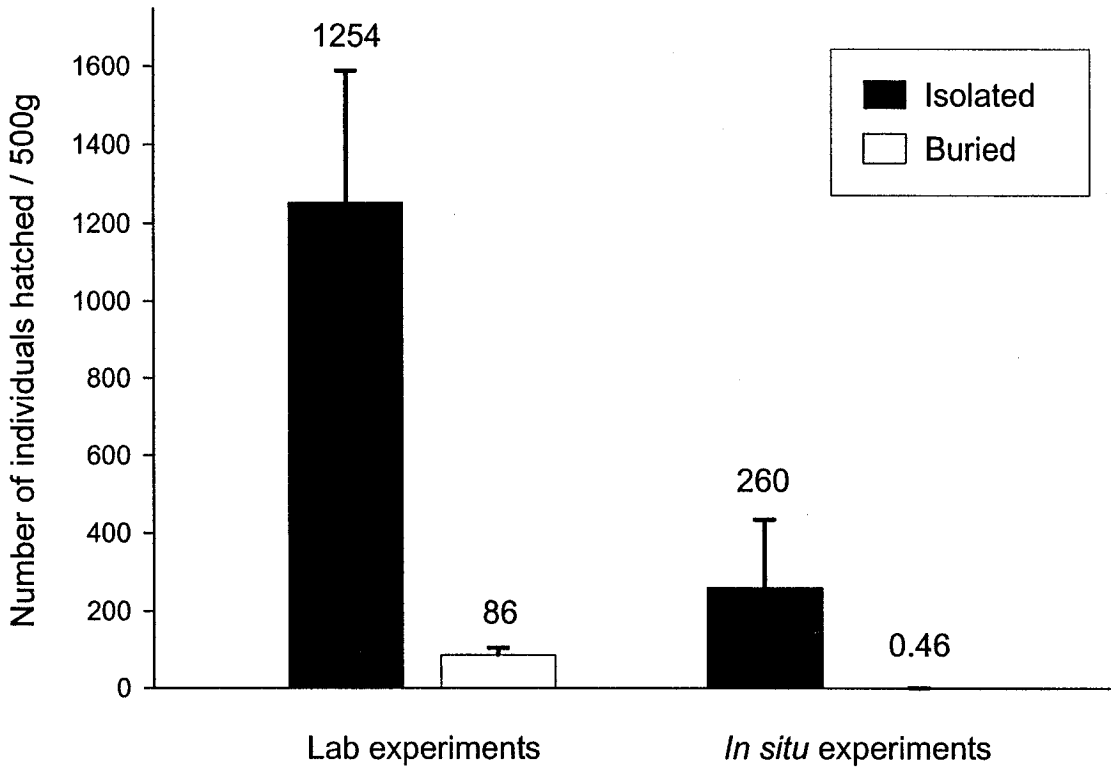

Figure 4.3 Total abundance of taxa hatched in laboratory and *in situ* studies from diapausing eggs isolated from, or buried in, residual ballast sediments.

SALINITY TOLERANCE OF DIAPAUSING EGGS OF FRESHWATER ZOOPLANKTON

5.1 ABSTRACT

Many freshwater zooplankton produce diapausing eggs capable of withstanding periods of adverse environmental conditions, such as anoxia, drought and extreme temperature. These eggs may also allow oligostenohaline species to survive increased salinity during periods of tidal flux or evaporation, and here I test the ability of diapause eggs to withstand such conditions. Salinity tolerance may also enable organisms to invade new environments. The increased rate of introduction of nonindigenous species to the Laurentian Great Lakes since 1989, when ballast water exchange regulations (to replace fresh/brackish water at sea with full seawater) were first implemented for transoceanic vessels, has stimulated studies that explore mechanisms of introduction, other than of active animals, in ballast water. One hypothesis proposes that freshwater organisms transported in ballast tanks as diapausing eggs are partially responsible for the increased rate of species introduction, since these eggs can tolerate a wide array of adverse environmental conditions. I collected ballast sediments from transoceanic vessels entering the Great Lakes, isolated diapausing eggs of three species (*Bosmina liederi*, *Daphnia longiremis* and *Brachionus calyciflorus*), and measured the effect of salinity on hatching rate. In general, exposure to salinity significantly reduced the hatching rate of diapausing eggs. However, since nonindigenous species can establish from a small founding population, the results from this study suggest that exposure to

high salinity water would not be a completely effective management tool for diapausing eggs.

5.2 INTRODUCTION

The introduction of nonindigenous species (NIS) is a potent agent of biodiversity change, particularly for lake ecosystems (Sala et al. 2000) and measures are urgently needed to identify and eliminate the vectors that transport them. Ballast water is recognized as the single most important vector for species introduction to aquatic habitats. Approximately ten billion tonnes of ballast water (and its associated biota) are transferred annually between global ports, providing the primary means of transport and introduction of nonindigenous aquatic biota to ecosystems, including bacteria, dinoflagellates, phytoplankton, zooplankton and fish (Rigby et al. 1999; Ruiz et al. 2000). Transoceanic shipping accounts for 77% of the species introduced to the Laurentian Great Lakes since 1970 (Ricciardi 2001). To reduce this threat, voluntary regulations were enacted in 1989, and mandated in 1993, that effectively require inbound vessels to exchange fresh or brackish ballast water with open-ocean saltwater if that water is to be discharged in the Great Lakes (USCG 1993). Despite these regulations, the rate at which new species have been recorded in the Great Lakes tripled between 1989 and 1999 compared with the preceding 40 years (Grigorovich et al. 2003a). Increased sampling effort and time lags between establishment and discovery of NIS may partially account for this pattern (Costello and Solow 2003; Grigorovich et al. 2003a), although modeling exercises indicated that ballast

water exchange offers only incomplete protection and is least successful for species with benthic or dormant stages contained within ballast sediments (MacIsaac et al. 2002b). Alternatively, the recent surge in NIS may be due to the presence of live or dormant organisms contained in the residual ballast of ships declaring 'no ballast on board', which are exempt from the regulations (MacIsaac et al. 2002b; Bailey et al. 2003; see Chapter II). These vessels carry tonnes of residual sediment and ballast water, and dominate trade inbound to the Great Lakes (Colautti et al. 2003).

Many freshwater zooplankton, including copepods, cladocerans and rotifers, produce diapausing or 'resting' eggs during annual population cycles. These dormant stages probably evolved as an adaptation to periods of adverse environmental conditions, including anoxia, drought and extremely low or high temperature (Gilbert 1974; Hairston 1996; Hairston and Cáceres 1996; Williams 1998). These eggs could also provide temporal escape from unfavourable salinity, facilitating the intercontinental transfer of freshwater species in sediments of ballast tanks, even those subjected to ballast water exchange. Some of the species recently recorded in the Great Lakes are euryhaline endemics of the Ponto-Caspian region of southeast Europe, which may have been transported as resting stages in ballast water and/or sediments (Ricciardi and MacIsaac 2000; Reid and Orlova 2002). While the salinity tolerance of some juvenile and adult freshwater cladocerans and rotifers has been examined (e.g., Miracle and Serra 1989; Teschner 1995; Hall and Burns 2002), very little is known of the salinity tolerance of the diapausing eggs of freshwater taxa.

Consequently, it is difficult to determine whether invertebrates capable of producing diapausing eggs could circumvent the salinity 'filter' imposed on potential Great Lakes invaders by ballast water exchange.

In this study, I examine the effect of salinity on the hatching rate of diapausing eggs of the cladocerans *Bosmina liederi* and *Daphnia longiremis*, and the rotifer *Brachionus calyciflorus*, common inhabitants of ballast sediments of transoceanic vessels entering the Great Lakes. While these three species are native to the Great Lakes, their presence in residual ballast sediments suggests that they are representative of the types of organisms that pose a potential risk of invasion. In Chapter II, I tested the viability of diapausing eggs recovered from ballast sediments and noted a tendency for reduced viability with high pore water salinity, although this relationship was not tested directly. Here, I test the hypothesis that diapausing eggs of freshwater zooplankton will be rendered non-viable by exposure to saline water.

5.3 METHODS

Sample collection — Residual sediments were collected from five transoceanic vessels entering the Great Lakes in 'no ballast on board' status in December 2000, May, August and December 2001, and in June 2002. Ships were sampled at the ports of Hamilton, Thorold and Toronto, Ontario, Canada, and Cleveland, Ohio, U.S.A. Detailed collection methods are described in Chapter II. The salinity of residual sediment pore water, separated from sediment by centrifugation at approximately 3 300 G (~32 360 $m \cdot s^{-2}$) for 15 min, was measured using an optical refractometer (F.C. Dobbs, Old Dominion University).

Egg Density Counts — After thorough mixing, four 40 g subsamples (wet weight) were taken from each sample and preserved in 95% ethanol. Subsamples were each washed through a 45 μm sieve to remove fine sediment. Diapausing eggs were subsequently separated from the coarse sediment using the colloidal silica Ludox® HS 40 (Burgess 2001) and counted under a dissecting microscope.

Hatching Experiments — Sediments were stored in plastic containers in the dark at 4°C for at least four weeks to allow a refractory period before hatching experiments commenced (see Grice and Marcus 1981; Schwartz and Hebert 1987). After this time, diapausing eggs were removed from sediment using a sugar flotation method (Bailey et al. 2003; Chapter II). Briefly, sediment was processed through a 45 μm sieve and washed into centrifuge tubes using a 1:1 (w:v) mixture of sucrose and water. After centrifugation (5 min at ~7.7 $m \cdot s^{-2}$) the supernatant was decanted and rinsed thoroughly with water through 45 μm mesh before being transferred to a counting dish. Diapausing eggs were immediately recovered from the supernatant and sorted by size and gross morphology under a dissecting microscope, selecting only fully intact, apparently healthy eggs. A single, replicated experiment was conducted on the most abundant egg type (*Brachionus* or *Daphnia* species) for each of five tanks. For sediments from ship 1, in which *Brachionus budapestinensis* eggs dominated (see Table 5.1), experiments were attempted using *B. budapestinensis*, but were abandoned owing to loss of eggs over time because of their extremely small size. Experiments were therefore conducted on a subdominant species, *Bosmina*

liederi. All other eggs were incubated at 0‰ for identification purposes only. Occasionally, two or three species hatched during a single trial (see Table 5.1). These secondary species always contributed less than 1% of the total number of hatchlings. In total, 11 species hatched, although only three were used in the replicated experiments. Four trials were conducted with the rotifer *Brachionus calyciflorus* and one trial each for the cladocerans *Daphnia longiremis* and *Bosmina liederi*.

Eggs used in the experiments were separated into 20 replicates of 20 eggs each, and placed into vials containing 15 ml of sterile medium (0, 8, 16, or 32‰) representing incremental efficiencies of ballast exchange. Five replicates were placed into each salinity treatment at 20°C (photoperiod 16 h light:8 h dark), resulting in an experimental design using 400 eggs per trial. The 0‰ treatment was considered a control to assess maximum viability for these freshwater species. Synthetic pond water (Hebert and Crease 1980) or diluted, filtered, natural seawater (collected from a vessel transiting the Great Lakes loaded with ocean water ballast, filtered through 0.2 μm Whatman number 5 paper filter) were used as hatching media. Vials were checked for emergence every 24 hours for ten days, with all hatched individuals removed daily. Media were refreshed on day five. On day 10 all remaining eggs were transferred to synthetic freshwater media by pipette to examine hatching rates after salinity exposure. Again, the number of hatchlings was recorded daily. Negative controls containing only treatment media were kept in each treatment group to detect any introductions of organisms from the environment. I chose the 10 day hatching period after

exposure for two reasons. First, previous experiments indicated that 96% of hatching occurs within the first 10 days of trials run for 20 or 30 days in the manner described above (Bailey et al. 2003; S.A. Bailey, unpubl.). Secondly, the typical transit time of a 'no ballast on board' vessel carrying Great Lakes water within the lake system is 7-10 days. If the uptake of Great Lakes water does induce diapausing eggs contained in ballast sediments to hatch (as demonstrated in Chapter IV), this is the period of greatest risk.

Variation in the cumulative proportion of diapausing eggs hatched between treatments was analysed using a one-way ANOVA with repeated measures using Systat 8.0 (SPSS Inc. 1998). Tukey's multiple comparison test was performed on the total proportion of eggs hatched to determine the impact of salinity on hatching rate. Since emergence was inhibited at higher salinities, analyses were conducted on the 10-day hatching segment for each treatment, depending on the timing of emergence (i.e., days 0-10 for 0 and 8‰ treatments were compared to days 10-20 for 16 and 32‰; if no hatching occurred during days 0-10 for the 8‰ treatment, then days 10-20 were used). Only days when hatching occurred in at least one of the replicates were analysed. The proportion of eggs hatched was normalised using an arcsine square root transformation before analysis.

5.4 RESULTS

Hatching experiments were conducted on zooplankton diapausing eggs isolated from residual ballast sediment collected from six tanks on five vessels.

Salinity of sediment pore water varied from 2‰ to 35‰ (Table 5.1). Diapausing egg densities of dominant taxa were high (> 50 eggs per 40 g sediment, Table 5.1). Eggs were induced to hatch in all experiments, with hatching rates ranging from 16 to 89% in the 0‰ treatment (Fig. 5.1). In each experiment the proportion hatching declined with increasing salinity. No organisms were recorded in the negative control vials. *Brachionus calyciflorus* generally began to hatch within 24 h of incubation at 0‰, while for the cladocerans *Bosmina liederi* and *Daphnia longiremis* emergence began at day 3 (Fig. 5.1). Development also began promptly in the 8‰ treatments, with *B. calyciflorus* hatching in three out of four trials by day 5. In addition, development of eye-stage embryos was recorded by day 5 in the 8‰ treatments for 50% and 90% of *Daphnia* and *Bosmina* eggs, respectively (see Fig. 5.2A). Apparently, these species could not tolerate emergence into brackish water, as development always stopped before emergence from eggs was complete. None of the eye-stage embryos recorded in 8‰ treatment continued development after the medium was replaced with 0‰ water on day 10. Conversely, no organisms hatched or completed significant development during the ten days of exposure at either of the two higher salinities (i.e., 16 and 32‰, Fig. 5.2B, C), although some lipid accumulation was noted in the 16‰ treatment. Rather, emergence occurred in these treatments only after brackish or saltwater media were exchanged for 0‰ water (Fig. 5.1). After exchange, hatching rates among experiments varied between 0-31% and 0-78% for the 16‰ and 32‰ treatments, respectively. *Bosmina liederi* was the only

species tested for which no hatching occurred after exposure to any of the salinity (> 0‰) treatments.

The difference in hatching rate between treatments was highly significant for all trials ($p<0.01$, ANOVA, Table 5.2). All trials exhibited divergence of hatching rates over time, as time*treatment interaction terms were significant ($p<0.0001$, ANOVA, Table 5.2). The proportion of eggs hatched was higher in the 0‰ treatment for eggs recovered from ships 1 to 4 (Fig. 5.1A-E; $p < 0.05$, Tukey *post hoc* test). The hatching rates of *B. calyciflorus* for the 0 and 32‰ treatments for ship 5 were significantly higher than for the other two treatments (Fig. 5.1F; $p<0.001$, Tukey *post hoc* test).

5.5 DISCUSSION

To date, investigations of the salinity tolerance of freshwater zooplankton have been limited to measuring direct effects on growth and survival (e.g., Miracle and Serra 1989; Teschner 1995; Hall and Burns 2002), or examining species richness and composition in waterbodies of varying salinity (Frey 1993; Brain et al. 1995). These approaches have not considered diapausing egg stages, probably resulting in an underestimate of the range of salinities a particular taxon can tolerate, particularly in instances where salinity varies temporally. In this study, I have demonstrated that the hatching rate of diapausing eggs is reduced by exposure to saline conditions. The ability of diapausing eggs to tolerate fluctuations in salinity may stem from an evolutionary

history in temporary habitats, which generally fluctuate more in their physical and chemical environment than adjacent, permanent ones (Williams 1998).

Of the species examined here, diapausing eggs of *Bosmina liederi* appear to exhibit the lowest salinity tolerance with no hatching after exposure to saltwater. *Daphnia longiremis* exhibited a modest degree of salinity tolerance, as a small proportion of diapausing eggs hatched following saltwater exposure. *Brachionus calyciflorus* demonstrated the widest tolerance, with up to 78% eggs hatching after saltwater exposure. Interestingly, *B. calyciflorus* also displayed a wider salinity tolerance as adults than either *B. liederi* or *D. longiremis*. Although typically considered to 'prefer' freshwater, *B. calyciflorus* is frequently observed in brackish waters (e.g., Brain et al. 1995; Park and Marshall 2000). It is also the only species in these trials that successfully hatched into 8‰ salinity during days 0-10, albeit at rates significantly lower than in freshwater. This finding is consistent with observations of Snell et al. (1991), who reported a ~40% reduction in the hatching rate of *B. calyciflorus* at 8‰ as compared to 2‰ growth media.

The 'maximum' hatching rates observed at 0‰ ranged from 16-89%. Results in Chapter II suggested that pore water salinity might be negatively correlated with hatching success. This study also found a low hatching rate for eggs recovered from sediments with a pore water salinity of 20‰ or higher (16 and 37%). However, lower pore water salinity (≤ 10‰) did not guarantee a high hatching rate (i.e., 32% hatch for 4‰), so other factors (such as age of eggs, duration of diapause or hatching cues) are probably involved. In addition, the

effects seen in both studies may be impacted by the wide salinity tolerance of *B. calyciflorus* (i.e., 10‰ is only slightly above the natural range for this species, resulting in a high hatching rate at intermediate levels of salinity). Alternatively, pore water salinity measured at the time of collection may not be a good indicator of egg history; eggs may have been retained in ballast sediments for years, experiencing widely varying salinity, of which only the most recent is reflected by pore water salinity. Nevertheless, while it is possible that the 'maximum' hatching rate (and subsequent reductions in hatching rate) measured in this study may be affected by previous exposures, these results do indicate the effectiveness of ballast water exchange because the sediments carried in transoceanic vessels originate from ports of varying salinity.

Hatching during days 10 to 20, following transfer to 0‰ medium, occurred mainly in the 16 and 32‰ treatments. Very little hatching occurred during this period in either the 0 or 8‰ treatments, with individuals emerging only from eggs that had not visibly developed during the first ten days. This suggests that the *Bosmina* and *Daphnia* eye-stage embryos that developed by day 5 at 8‰ were no longer viable. It is possible that a salinity of 8‰ is sufficiently low for the initiation of egg development in freshwater species, but too high for complete development and emergence to occur. In contrast, no development in these genera was apparent in eggs exposed to 32‰ water, thus there remained a 'bank' of viable embryos left to emerge following transfer to freshwater media. Although both *B. liederi* and *D. longiremis* displayed this trend, I cannot explain why only *Daphnia* eggs hatched after exposure to 32‰. However, this trend was

also observed for *B. calyciflorus*, with a higher emergence rate after exposure to higher rather than to lower salinity during the latter half of the experiment, particularly for eggs from ship 5. A similar phenomenon was observed by Lutz et al. (1994), who exposed copepod resting eggs to variable oxygen conditions. These authors noted that low oxygen concentrations were more detrimental to egg viability than total anoxia because metabolism was completely shut down during anoxia but not under low oxygen conditions. Thus, there appears to be greater interaction between the embryo and the environment under nearly favourable conditions than under extreme conditions. However, it is also possible that the transfer of eggs from 32‰ to 0‰ acted as a stronger hatching cue than the transfer of eggs from 8 or 16‰ to 0‰. If this is the case, then subjecting diapause eggs to ballast water of 32‰ may actually promote mass hatching once the eggs are returned to freshwater conditions.

Charmantier and Charmantier-Daures (2001) suggested that rehydrated *Artemia* embryos are protected from high salinity by the cyst envelope that is permeable to water but impermeable to ions. However, salinity and temperature are known to interact in their effects on tolerance, with temperature affecting metabolic rate, ion uptake rate, and membrane permeability (Lee and Bell 1999). These experiments explored salinity tolerance at 20°C, arguably a more challenging environment than exposure at a lower temperature for temperate species. It will be necessary to conduct future trials at a variety of temperatures to deduce the interaction between temperature and salinity on diapausing egg viability.

The variation in hatching rate seen among *Brachionus calyciflorus* trials after exposure (day 10-20) may have resulted from the disparate histories of the populations tested, as indicated by pore water salinity of ballast sediments. Of particular interest was the hatching rate of *B. calyciflorus* collected from ship 5, as 78% eggs hatched successfully after exposure to salinities up to open-ocean levels (i.e., 32‰). In contrast, hatching rates of the other three *B. calyciflorus* populations were significantly reduced after similar exposure (<10%). It is possible that salinity experienced during diapause egg formation influences the range of salinities eggs can survive while dormant, much like it affects the optimal salinity for the initiation of hatching for the euryhaline rotifer *Brachionus plicatilis* (Gilbert 1974). I was unable to explore this hypothesis, as the origins of the diapausing eggs in this study are unknown. Future studies using clonal populations from both permanent and temporary habitats will help clarify this possibility.

In general, less than 10% of *Daphnia* and *Brachionus* eggs hatched after salinity exposure in these experiments. Nevertheless, considering the high egg density in ballast sediments (10^4 to 10^5 eggs·m^{-2} using 1.6 g·cm^{-3} conversion factor for wet sediment), large populations of viable zooplankton eggs may remain after salinity exposure. While it is possible that a longer exposure would have reduced egg viability further, the length of transoceanic crossings will generally not permit longer exposure regimes. Since only a small 'seed population' is necessary to establish a cohort of reproductive individuals, and given that the maximum density of diapausing eggs in natural populations ranges

between 10^3 and 10^6 eggs·m^{-2} (Hairston 1996), new populations could establish when salinity returns to favourable values (i.e., when the vessel subsequently loads freshwater, or if the eggs get flushed into a freshwater environment). Hall and Burns (2001) suggest that resting eggs of *Boeckella hamata*, a freshwater copepod, are responsible for the recolonization of the tidally-influenced Lake Waihola, New Zealand, after seasonal salinization up to 4.8‰. The average hatching rate for resting eggs of *B. hamata* was only 2.3% under optimal conditions in the laboratory. Therefore, while ballast water exchange may reduce the viability of diapausing eggs by as much as 90% for some taxa, it apparently does not offer complete protection against NIS entering the Great Lakes by this mechanism. Interestingly, this study indicates that ballast water exchange using brackish water (e.g., 8‰) may have a larger impact on diapausing egg viability than 32‰; however, this effect would have to be weighed against the possibility of introducing live euryhaline species in water of lower salinity, for which ballast water exchange of 32‰ is decidedly more effective (Locke et al. 1993; MacIsaac et al. 2002b).

Furthermore, most transoceanic vessels currently trading on the Great Lakes declare 'no ballast on board' status (Colautti et al. 2003), and thus are exempt from ballast water exchange regulations (USCG 1993). MacIsaac et al. (2002b) suggested that these vessels, collectively, may pose a higher invasion risk than vessels entering the system with saline ballast water owing to the abundance of viable diapausing eggs contained within residual sediments. These results suggest that the risk posed by diapausing eggs present in sediments of

these vessels could be reduced, but not eliminated, by introducing a lens of saltwater into the 'empty' ballast tanks similar to ballast exchange.

Sala et al. (2000) suggested that lakes will experience very steep declines in biodiversity this century owing to biotic exchange, land use change and climate change. The salinity of endorheic freshwater habitats is likely to increase during summer months as water inputs decline and evaporation increases (Schindler 1997, 2001). In addition, coastal lakes and freshwater habitats upstream from tidal estuaries may suffer periodic salinization as pulsing surges of saltwater seep inland owing to evaporation and anthropogenic diversion of freshwater (Jones 1994; Hall and Burns 2003). The persistence of populations through salinity fluctuations by means of diapausing eggs could have profound implications on the extent of biodiversity loss during habitat change. Species incapable of tolerating changing salinity could be replaced by taxa tolerant of brackish or saline conditions (Schindler 1997); however, this study demonstrates that some populations may be capable of tolerating enhanced fluctuations in habitat salinity, providing a mechanism for enriching biodiversity if the habitat returns to freshwater conditions.

Table 5.1 List of species hatched from ballast sediments through quantitative and qualitative hatching studies.

Ship	No. of replicates	Species	No. eggs per 40 g	Pore water salinity (‰)
1 (FP)	N/A	*Brachionus budapestinensis*	92	2
	5	*Bosmina liederi*	56	
	N/A	*Brachionus calyciflorus*	52	
	N/A	*Daphnia longiremis*	6.3	
	N/A	*Daphnia ambigua*†		
2 (DB)	5	*Daphnia longiremis*	391	10
	N/A	*Daphnia ambigua*†		
3 (DB)	5	*Brachionus calyciflorus*	100	35
	N/A	*Brachionus quadridentatus* f. *rhenanus*†		
	N/A	*Brachionus urceolaris*†		
	N/A	*Brachionus budapestinensis*	56.5	
	N/A	*Brachionus angularis* Gosse†		

Table 5.1 (continued)

4 (DB)	5	*Brachionus calyciflorus*	57.8	4
	N/A	*Daphnia magna*	1.5	
	N/A	*Diaphanosoma* sp.	<1	
4 (FP)	5	*Brachionus calyciflorus*	119.5	20
5 (DB)	5	*Brachionus calyciflorus*	187.8	10
	N/A	*Asplanchna brightwelli*	1.5	

Note: Species with N/A replicates were not used during experimentation, and were hatched only for identification purposes. Ship tanks are identified by type: FP = forepeak tank, DB = double-bottom tank. †denotes secondary species hatched from single morphological egg type listed immediately above.

Table 5.2 ANOVA with repeated measures demonstrating the effect of salinity treatment on the hatching rate of diapausing eggs.

		ANOVA effects *F* value (df)		
Ship	Organism	treatment	time	time * treatment
1 (FP)	*Bosmina liederi*	32.56*** (3,16)	43.48*** (3,48)	16.98*** (9,48)
2 (DB)	*Daphnia longiremis*	368.97*** (3,16)	353.05*** (9,144)	350.90*** (27,144)
3 (DB)	*Brachionus calyciflorus*	19.78*** (3,17)	41.08*** (7,119)	10.39*** (21,119)
4 (DB)	*Brachionus calyciflorus*	36.60*** (3,16)	47.26*** (4,64)	15.10*** (12,64)
4 (FP)	*Brachionus calyciflorus*	6.15* (3,16)	14.97*** (6,96)	6.54*** (18,96)
5 (DB)	*Brachionus calyciflorus*	35.25*** (3,16)	322.04*** (4,64)	22.47*** (12,64)

Note: Data were arcsine square root transformed prior to analysis. Significance levels for *F*-values: * ($p<0.05$), *** ($p<0.0001$). Ship tanks are identified by type: FP = forepeak tank, DB = double-bottom tank.

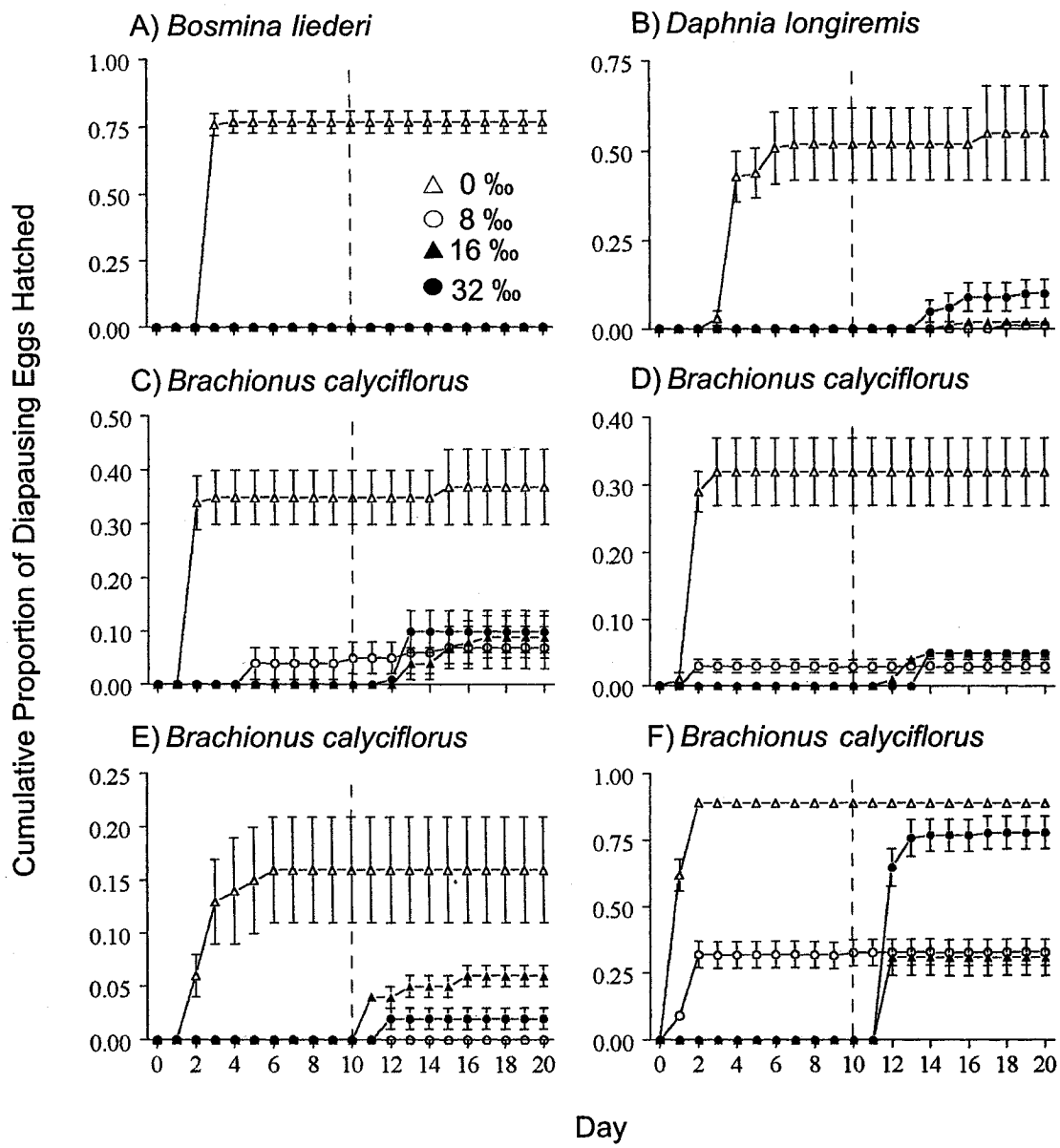

Figure 5.1 Mean (± S.E.) cumulative proportion of diapausing eggs hatched under salinity treatments, by species. A) *Bosmina liederi* (ship 1), B) *Daphnia longiremis* (ship 2), C) *Brachionus calyciflorus* (ship 3), D) *B. calyciflorus* (ship 4-DB), E) *B. calyciflorus* (ship 4-FP), *F*) *B. calyciflorus* (ship 5). After 10 days (dotted vertical line) all unhatched eggs in each treatment group were transferred to 0‰ media. Note scale difference for each ordinate. Error bars less than 0.03 are hidden by graph symbol.

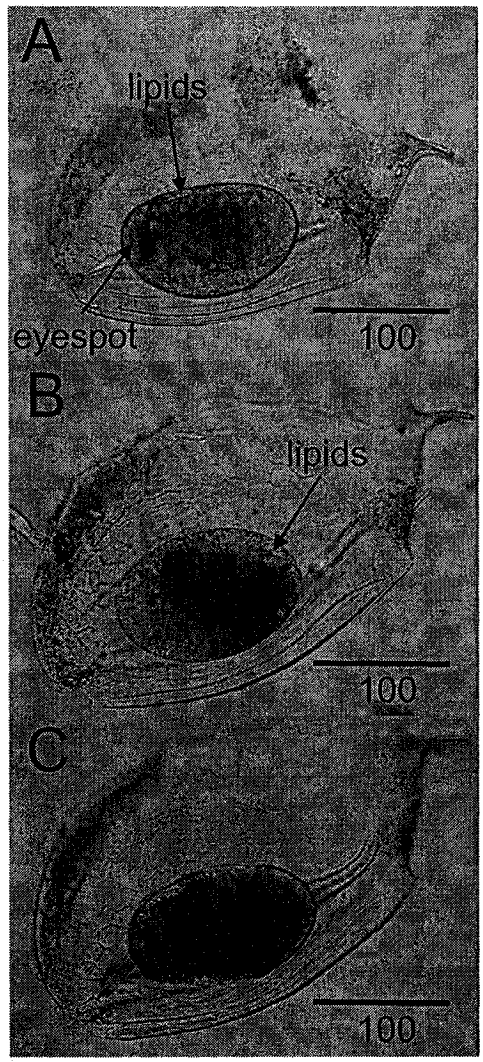

Figure 5.2 Condition of diapausing eggs of *Bosmina liederi* after 10 days at each treatment. A) 8‰, aborted eyed-embryo, B) 16‰, little differentiation, C) 32‰, no change. Scale bars (100 μm) are included on each image.

GENERAL DISCUSSION

Laboratory hatching studies that characterized the density, diversity and viability of invertebrate resting stages present in ballast sediments of transoceanic vessels support the hypothesis that residual sediments transport new species to the Laurentian Great Lakes (and elsewhere). Furthermore, *in situ* experiments suggest that invertebrates transported as resting stages can be induced to hatch inside ballast tanks under operational conditions, providing an opportunity for introduction of planktonic individuals when ballast water is subsequently discharged. Dormancy is a commonly encountered life history strategy in freshwater invertebrates that ensures long-term survival through adverse conditions (see Bilton et al. 2001). Resting stages, which can survive periods of anoxia, desiccation and fluctuating temperature (Dodson and Frey 2001; Wallace and Snell 2001; Lutz et al. 1992), are also essential for natural dispersal. Locke et al. (1991) first speculated that resting stages may be carried in ballast sediments; these authors hypothesized that resting stages make good invaders because they are less likely to get swept out of tanks during ballast exchange, and because they may survive the associated salinity shocks.

There are several entry mechanisms associated with transoceanic shipping that can introduce NIS to the Great Lakes: hull fouling, ballast water and/or sediments contained in ships carrying 'ballast on board' (BOB), and ballast water and/or sediments contained in ships declaring 'no ballast on board' (NOBOB); the sediment vectors can further be subdivided into active or dormant individuals present in sediments. It is difficult to determine the relative importance of ship-

related mechanisms as many established NIS could have been introduced via multiple pathways and as there have been few comprehensive studies of these vectors (Holeck et al. 2004). Despite carrying larger volumes of ballast water, ships in BOB status probably present a lower risk of introduction of NIS than NOBOB vessels because the latter constitute approximately 90% of vessel traffic operating on the Great Lakes (Colautti et al. 2003). In addition, hull fouling and (exchanged) ballast water of BOB ships are probably relatively minor vectors, since freshwater taxa that could survive in the Great Lakes likely could not withstand the high salinities encountered during a transatlantic voyage. Although early studies suggested freshwater taxa may be able to survive incomplete ballast exchange (Locke et al. 1991; 1993), recent work suggests that ballast exchange is usually comprehensive (95-99%; Ruiz et al. 2004). Thus, presumably only small numbers of euryhaline taxa could exploit these vectors for entry.

In terms of ballast sediments, propagule loads of invertebrates (active and dormant stages) should be similar on individual BOB and NOBOB vessels, assuming that they operate in similar trade areas. Again, however, far greater numbers of NOBOB vessels discharge ballast water in the Great Lakes than BOB vessels. My results indicate that the median density of dormant invertebrates transported by NOBOB vessels, 7.2×10^5 ship^{-1}, is approximately half that of active animals present in sediments (1.3×10^6 ship^{-1}; Duggan et al. in review; Table 6.1). Furthermore, a larger proportion of epibenthic taxa are expected to be discharged with ballast water than are individuals hatched from

resting stages (I.C. Duggan, University of Waikato, pers. comm.; Chapter IV), indicating that active invertebrates associated with sediments pose a greater risk of invasion. Despite having densities an order of magnitude lower than that of dormant stages (1.1×10^4 ship^{-1}; Duggan et al. in review; Table 6.1), active invertebrates transported in residual water also appear to pose a greater invasion risk. Whereas *in situ* hatching studies indicate that less than 1% of diapausing eggs will hatch under normal operational conditions (Chapter IV), 99% of active taxa present in residual water may be discharged (MacIsaac et al. 2002b), indicating that the total number of propagules introduced from residual water nearly exceeds that of dormant stages by a factor of 100. One caveat, however, is that invertebrates hatched from diapausing eggs are parthenogenetic and thus have a greater potential for increases in inoculum size due to reproduction than active, sexually-reproducing, animals in either residual water or sediments.

Although the risk of invasion associated with resting stages in ballast sediments appears to be lower than that of active animals in residual water and sediments, it clearly is greater than zero. Since only a small "seed population" is necessary to establish a viable population, particularly of asexual species (Drake 2004), I investigated the efficacy of saltwater exposure as a tool to reduce viability of diapausing eggs. In general, less than 10% of diapausing eggs hatched after exposure to salinities of 8, 16 or 32‰. However, the remaining eggs could hatch and potentially establish new populations when salinity returns to favourable values (i.e., when the vessel subsequently loads freshwater, or if the eggs get flushed into a freshwater environment). Therefore, ballast water

exchange apparently does not offer complete protection against NIS entering the Great Lakes as diapausing eggs. Surprisingly, this study indicated that ballast water exchange using brackish water (e.g., 8‰) may have a larger impact on viability of diapausing eggs than does 32‰; however, the utility of this management strategy would have to be weighed against the possibility of introducing live euryhaline species in water of lower salinity, for which ballast water exchange of 32‰ is decidedly more effective (Locke et al. 1993; MacIsaac et al. 2002b).

Measures of total propagule load likely overestimate the invasion risk posed by shipping vectors, as many of the transported taxa may not be able to tolerate abiotic conditions of, or will be cosmopolitan species that are already distributed in, the recipient region. Locke et al. (1993) found a low occurrence of species considered nonindigenous to the Great Lakes in ballast water of BOB ships (4 of 57 species). Similarly, this dissertation indicates that both abundance and occurrence of viable resting stages of NIS transported in residual sediments of NOBOB ships are low (approximately 2.5 and 32%, respectively). In total, I identified 78 distinct taxa from resting stages in residual sediment, with nearly the entire assemblage representing planktonic freshwater species, particularly rotifers. Although 70% of species encountered during this study are considered native to the Great Lakes and do not appear to represent an invasion risk, native species could suffer reduced population fitness via outbreeding depression if novel genotypes are introduced from global ports (e.g., Saltonstall 2002; Turon et al. 2003; see Rhymer and Simberloff 1996). Furthermore, the possibility exists

that some Nearctic species could be transferred from the Great Lakes to trading partners overseas (e.g., *Kellicottia bostoniensis* introduced to Sweden; Josefsson and Andersson 2001). This possibility highlights the fact that invasions are a global phenomenon.

My hatching studies suggest that rotifers present the predominant invasion risk to the Great Lakes via diapausing eggs in sediments since they occur in the highest frequency and abundance, and as they were the predominant taxa hatched during *in situ* hatching trials. Cladocerans, and to a lesser extent copepods, may also be introduced via resting stages in residual sediments. Although recent collection of nonindigenous rotifer species in the Great Lakes are an indication that introduction of new rotifers already have occurred (Hwang and Heath 1999; Gray et al. 2005), successful rotifer invasions have not been reported for the Great Lakes. The lack of established populations of NIS by this group indicates that resting stages contained in residual sediments are a weak or emerging vector.

While dormancy is a characteristic that enables enhanced survival during transportation, it becomes an impediment to introduction, as less than 0.05% of individuals will likely pass from the transportation stage to the introduction stage under conditions experienced in ships' ballast tanks. As environmental and demographic stochasticity will further reduce the number of individuals successfully transitioning from the introduction stage to the establishment stage of the invasion process, the risk of invasion via diapausing eggs in residual ballast sediments appears to be low. However, the assumption that ballast

sediments are not being deposited directly into the Great Lakes, either during regular deballasting or tank cleaning operations, must be validated to ensure that this risk is not underestimated. Dry-dock cleaning of sediments from ballast tanks should be carefully managed, since it can provide a direct route for discharge of diapausing eggs into adjacent waterbodies.

Table 6.1 Comparison of invasion risk factors for NOBOB sub-vectors. Data for active invertebrates in residual water and sediment provided by I.C. Duggan, University of Waikato, pers. comm. and in Duggan et al. in review. Data for residual sediment (dormant) compiled from Chapters III and IV. * denotes number of individuals available from hatching *in situ* and does not include any increase in inoculum size through reproduction.

Sub-vector	Volume per ship (tonnes)	Density per tonne	Proportion of freshwater NIS (%)	Probability of discharge (%)	Propagules freshwater NIS per ship
Residual water (active)	~47	1.1×10^4	~1.2	~99	~6,150
Residual sediment (active)	~15	1.3×10^6	~0.6	~8	~9,350
Residual sediment (dormant)	~15	7.2×10^5	~1.7	~0.05	~90*

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APPENDIX

List of invertebrate taxa hatched from resting stages during this study, arranged taxonomically. Occurrence lists number of ships that the species was collected on, from possible 35. Abundance lists the range (median) number of individuals emerging from 40 g sediment for all ships. Experiment type lists presence of species in maximum diversity (isolated from sediment) and whole sediment (buried in sediment) trials. All species able to hatch in 0‰ medium unless otherwise indicated: †denotes exclusively in 8‰; ‡denotes exclusively in 32‰.

Taxon	Occurrence	Abundance	Experiment Type	
			Maximum Diversity	Whole Sediment
Gastrotricha				
Chaetonotidae, unidentified	1	3	X	
Rotifera				
Ascomorpha ecaudis	1	0.25	X	
Ascomorpha saltans	1	0.25	X	
Ascomorpha sp.	1	1	X	
Asplanchna brightwelli	4	0.25-1 (0.75)	X	X
Asplanchna girodi	2	0.5 (0.5)	X	X
Asplanchna priodonta	3	1-11.5 (3)	X	X

Brachionus angularis	21	0.25-21.8 (4)	X	X
Brachionus bennini	1	0.25	X	
Brachionus budapestinensis	15	0.75-341.5 (3)	X	X
Brachionus calyciflorus	25	0.63-77.77 (3)	X	X
Brachionus caudatus	2	0.5-2 (1.25)	X	
Brachionus diversicornis	1	0.25	X	
Brachionus forficula	1	1	X	
Brachionus havanaënsis	2	1 (1)	X	
Brachionus leydigi	4	0.25-1 (0.78)	X	X
Brachionus nilsoni	1	1	X	
Brachionus quadridentatus	4	0.5-12.25 (1.25)	X	X
Brachionus urceolaris	9	0.25-78 (1)	X	X
Cephalodella catellina	2	0.25 (0.25)	X	
Cephalodella forficula	1	0.25	X	
Cephalodella cf. *stenroosi*	1	0.3		X
Cephalodella sterea	1	4.75	X	
Cephalodella cf. *theodora*	1	0.25	X	
Cephalodella sp.	1	1	X	
Conochilus coenobasis	1	0.5	X	
Conochilus dossuarius	1	1	X	

Conochilus hippocrepis	2	1 (1)	X	
Conochilus cf. *natans*	1	0.25	X	
Conochilus unicornis	1	0.8		X
Dicranophoridae, unidentified	1	83	X	
Euchlanis cf. *dilatata*	2	0.25-1 (0.63)	X	
Filinia brachiata	1	0.25	X	
Filinia cornuta	3	0.5-1 (0.5)	X	
Filinia longiseta	6	0.25-4 (1)	X	
Filinia passa	4	0.25-1 (0.75)	X	
Filinia terminalis	5	0.38-2.5 (1)	X	
Flosculariidae, unidentified	1	0.25	X	
Hexarthra intermedia	1	0.25	X	
Hexarthra mira	3	0.25-1 (1)	X	
Keratella cochlearis	3	0.25-1 (1)	X	
Keratella quadrata	5	0.25-4 (0.5)	X	X
Keratella tropica	1	2	X	
Keratella sp.	1	1	X	
Lacinularia sp.	1	0.25	X	
Lecane closterocerca	2	0.3-0.5 (0.4)	X	X
Lecane flexilis	1	0.25	X	

Lindia truncata	1	0.5	X	
Ploesoma truncatum	3	0.25-2 (2)	X	
Polyarthra dolichoptera	9	0.5-5 (1)	X	
Polyarthra vulgaris	6	0.25-21 (2)	X	
Polyarthra spp.	2	0.25-1 (0.63)	X	
Pompholyx sulcata	4	0.25-7 (3.5)	X	
Synchaeta bacillifera	1	2.25	X†	
Synchaeta baltica	1	2.75		X‡
Synchaeta kitina	1	0.25	X	
Synchaeta oblonga	1	0.25	X	
Synchaeta stylata	4	0.25-1 (0.28)	X	X
Synchaeta tremula	2	1-3.5 (1)	X	X
Synchaeta sp.	1	0.25		X†
Trichocerca multicrinis	1	39	X	
Trichocerca pusilla	7	1-17.63 (1.25)	X	
Trichocerca rattus	1	1	X	
Trichocerca similis	1	0.25	X	
Monogonont, unidentified	2	1 (1)	X	
Bryozoa				
Plumatella casmiana	2	0.25-1 (0.63)	X	

Plumatella sp.	1	0.25	X	
Anomopoda				
Alona rectangula	1	0.5	X	
Alona rustica	1	0.25	X	
Bosmina liederi	3	1-6 (1)	X	
Bosmina maritima	1	2	X	
Bosmina spp.	2	1 (1)	X	
Ceriodaphnia quadrangula	1	0.25	X	
Ceriodaphnia sp.	2	1 (1)	X	
Daphnia longiremis	2	1 (1)	X	
Daphnia magna	4	0.5-2 (1)	X	X
Daphnia retrocurva	1	2	X	
Disparalona leei	1	0.25	X	
Moina micrura	2	1-47.88 (24.44)	X	
Moina sp.	1	1	X	
Ctenopoda				
Diaphanosoma birgei	2	0.75-6 (3.38)	X	
Diaphanosoma brachyurum	1	0.25	X	
Diaphanosoma mongolianum	1	0.5	X	
Diaphanosoma orghidani	1	1.25	X	

Diaphanosoma sarsi	1	0.25	X	
Diaphanosoma spp.	6	1 (1)	X	
Onychopoda				
Evadne nordmanni	1	0.5	X[†]	
Copepoda				
Acanthocyclops robustus	1	0.8		X
Cyclopoida, unidentified	3	0.25-1.25 (0.25)		X
Nitocra lacustris	1	1		X
Copepod nauplii, unidentified	14	0.25-20 (3)	X	X

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